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**THE INFLUENCE OF pH ON THE SURVIVAL AND PATHOGENICITY OF
SALMONELLA ENTERITIDIS PHAGE-TYPE 4**

by

ANN SHEENA MC DERMID B.Sc. (Hons.)

A thesis submitted in partial fulfilment of the requirements
of the Open University for the degree of

DOCTOR OF PHILOSOPHY

August 1998

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The influence of pH on the survival and pathogenicity of

***Salmonella enteritidis* PT4**

Ann S. McDermid

Abstract

An ability to survive the sometimes hostile conditions encountered in food, the environment or a host will be crucial to the pathogenicity of *Salmonella enteritidis* PT4. Isolates of *S. enteritidis* and *S. typhimurium* generally grew under a wider range of stresses when compared to other serotypes of *Salmonella*; they also survived well in aerosols. *S. enteritidis* PT4 demonstrated enhanced pH tolerance.

Adverse pH was an important discriminatory factor; as this is frequently experienced in nature, the effect of pH on the physiology, tolerance, pathogenicity and proteome expression of a clinical isolate of *S. enteritidis* PT4 was investigated further. The pH limits for continuous growth (MGT=6.9h), in a minimal medium supplemented with mucin were pH 4.35 and pH 9.45. The adaptive responses of acid and alkaline grown cells differed. Generally, acid grown cells were more tolerant, demonstrating cross-protection to a range of stress factors. In contrast, growth at pH 9.45 induced sensitivity to some stress factors. Growth at the pH limits reduced the percentage of cells expressing fimbriae and/or flagella, and the carriage of the 38MDa plasmid. Cells grown at pH 4.35 were significantly more virulent, both in terms of the number of deaths and the time to death in a mouse model, when compared to pH 7.10 or pH 9.45 grown cells.

The proteome of *S. enteritidis* varied with growth pH when assayed by two-dimensional electrophoresis with N-terminal sequence analysis. The changes were predominantly quantitative with few novel proteins being observed. Proteins which were regulated in response to growth pH included the *S. enteritidis* homologues to enolase, GroEL and a precursor of the leu/ileu/val binding protein of *S. typhimurium* or *Escherichia coli*.

The pH adaptive responses of *S. enteritidis* PT4 may have a significant role in the life of the cell, by influencing physiology, metabolism, viability and pathogenicity for mice.

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Chapter 1

Background

1.1 The evolution of microbiology and epidemiology

Long ago, the spread of disease from one person to another suggested the existence of invisible, transmissible agents of infection. In the poem *De rerum natura*, Lucretius (~96-55 B.C.) recognised the existence of “seeds” of disease. Epidemiological studies from as early as 1546 by Fracastorius of Verona presented evidence on the spread of plague and syphilis. He postulated that the disease agent was spread from one person to another directly or via transfer on an inanimate object, and this was a novel idea at this time. However, organisms were not seen until Antonie van Leeuwenhoek (1632-1723) made microscopes with adequate magnification. With the tiny ground lenses he made, Leeuwenhoek was able to observe and differentiate different types of bacteria including spheres, rods, and spirals. The field of microbiology began with his letter in the *Philosophical Transactions of the Royal Society of London* in 1677.

The idea of spontaneous generation persisted for microbes until the concept of sterility was first investigated by Spallanzani (1729-1799) and, subsequently, by Louis Pasteur (1822-1895). Once the biological continuity of microbes was recognised, the recovery of different bacteria from divergent environmental niches soon followed. The advent of selective culture techniques and media led to the identification of bacteria with different properties and eventually to the classification of bacteria into families, genera and species.

1.2 *Salmonella*

Salmonella were first isolated and identified as pathogens more than a century ago; the first isolation was made in 1885 by an American veterinarian, D.E. Salmon. *Salmonellae* are ubiquitous, and can be isolated from the gut contents

of animals, wild birds, domestic pets and rodents. They are primarily animal pathogens which occasionally infect man. People have probably been infected by *Salmonella* for thousands of years by living in close contact with their animals, butchering them and consuming their flesh or eggs, and from faecally-contaminated water (Karlen 1995). Neolithic man was exposed to a stream of new pathogens as birds and pigs scavenged around their camps and villages. New diseases of man emerged as new species of animals and birds became domesticated.

The salmonellae are members of the family Enterobacteriaceae: they are Gram-negative, usually motile (although non-flagellate types occur) and some serotypes are characteristically non-motile (Cowan and Steel 1974). Originally *Salmonella* species were named according to the disease they caused or the animals from which they were first isolated. Later, each antigenically distinguishable type was named after the geographical place at which it was first isolated.

In 1934, the *Salmonella* Subcommittee decided that serology was the ultimate criterion in the classification of the group, and in excess of 2,200 serotypes have been described. The serological division is based on differences in the O (somatic), Vi (capsular) and H (flagellar) antigens using the Kauffmann-White scheme. As the number of isolates increased, serotyping alone became inadequate for epidemiological purposes and a phage-typing scheme for *S. enteritidis* was developed in 1987 (Ward *et al.* 1987). This gives further differentiation based on the patterns of sensitivity to sets of typing bacteriophage. This scheme has proved invaluable as a phenotypic subtyping method and improves discrimination between strains. The original scheme contained 10 typing phages, defining 27 distinct patterns. This has since been extended to include 15 typing phages for differentiating a greater number of phage-types (Stanley and Baquar 1994). This level of differentiation is far from being a purely academic exercise, but greatly increases the precision with which the sources and methods of spread of outbreaks can be traced.

It is now widely agreed that the member 'species' of the genus *Salmonella* are actually a single species when characterised by the conventional criteria of bacterial taxonomy. The primary species of the genus *Salmonella* has been named *Salmonella enterica* with the strain used in this study being properly designated *Salmonella enterica* serotype Enteritidis (Selander *et al.* 1996). The older nomenclature has prevailed and this strain is regularly referred to in the form: *S. enteritidis*, *S. Enteritidis* and *Salmonella enterica* serotype Enteritidis. For convenience, I have selected to use the epithet *S. enteritidis*.

Molecular biological techniques, such as plasmid profiling and ribotyping have all been investigated as epidemiological markers. However, the reliability of these methods has been hampered by low strain discrimination. The use of PCR ribotyping in molecular subtyping of strains of *S. enteritidis* has been used successfully in some countries (Nastasi and Mammina 1996).

S. enteritidis has been shown to be closely related to other serogroup D serotypes: *S. dublin*, *S. gallinarum* and *S. pullorum* (Stanley and Baquar 1994). Using four different chromosomally-based typing methods (ribotyping, random cloned chromosomal probes, pulsed-field gel electrophoresis and probing with IS200), phage-type 4 showed the same characteristics as phage-types 4a, 6, 7, 21 25 and 31 (Olsen *et al.* 1994b). The close relationship of PT4 and PT7 is supported by the evidence that *S. enteritidis* PT4 converts to PT7 when LPS is lost (Threlfall *et al.* 1993).

1.3 Host specificity

Most *Salmonella* serotypes are host specific, being more virulent for the species to which they are adapted:- *S. typhi* causes human typhoid fever, *S. choleraesuis* causes disease in pigs, *S. dublin* typically produces systemic infection and gastroenteritis in cattle and to a lesser extent in sheep and man, *S. abortusovis* is associated with sheep, while in avian hosts, *S. gallinarum* and its biotype *S. pullorum* produce the most widespread disease (Barrow *et al.* 1994). The reasons for such host specificity are unclear, although the findings that *S. typhimurium* and *S. typhi* target different epithelial-cell receptors for

translocation into the gastrointestinal mucosa may contribute to their different host ranges and disease symptoms (Pier *et al.* 1998).

In contrast, *S. typhimurium* and *S. enteritidis* can produce systemic disease in a wide range of animal species (Old 1990). These serotypes do not routinely cause systemic disease in immunologically-mature healthy adult animals or man; however, they are capable of producing such disease in the young, very old or in those who are immunologically or otherwise compromised.

1.4 Why Study *Salmonella enteritidis* PT 4 ?

Throughout evolution, *S. enteritidis*, like many other microbes, has evolved to be a successful pathogen, being able to survive and proliferate rapidly under a wide range of environmental conditions. The kinds of strategies developed by bacteria for enhanced survival and competitiveness are shown in Fig. 1.1.

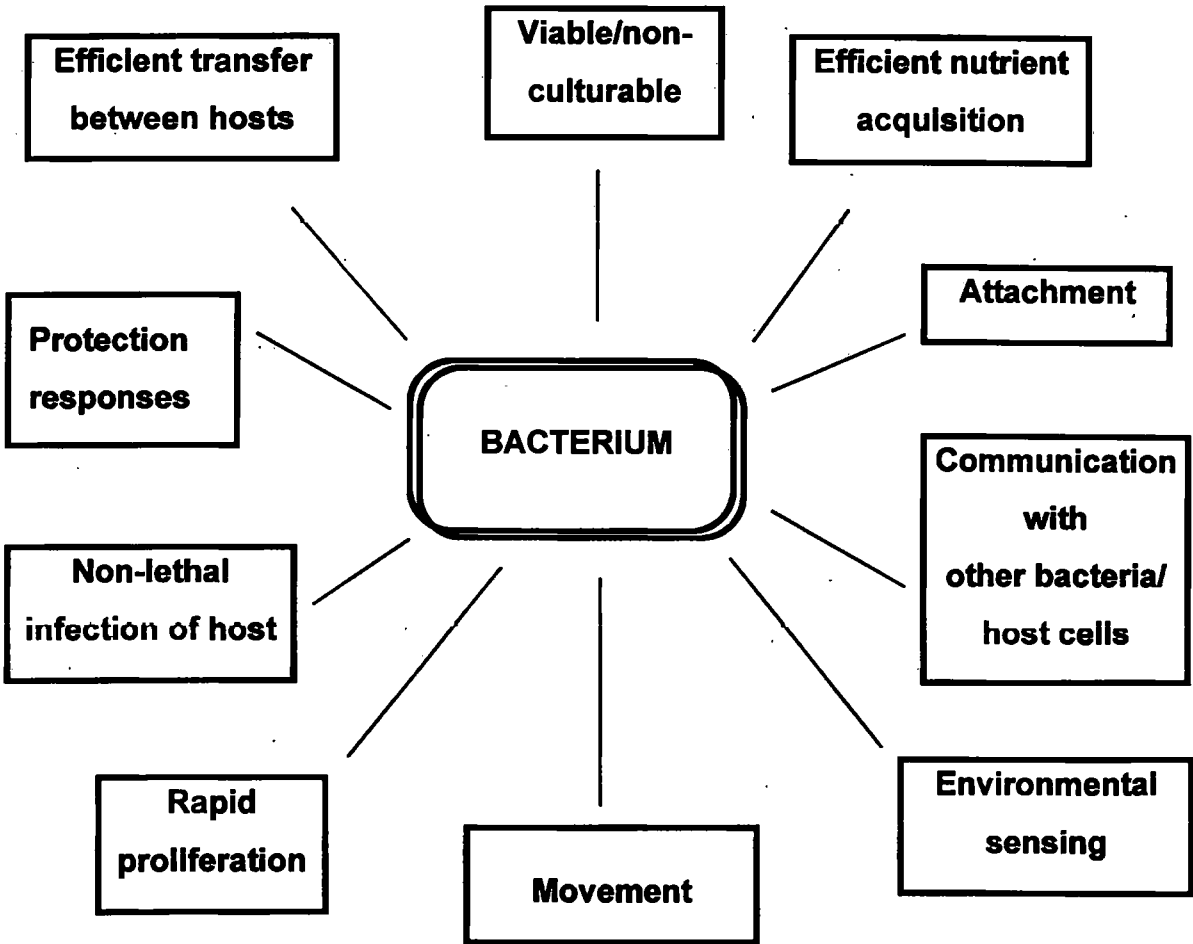


Figure 1.1 Mechanisms evolved for increased competitiveness of bacteria

The relevance and nature of some of these properties in *S. enteritidis* PT4 are undetermined. The mechanisms which enable *S. enteritidis* PT4 to survive in a range of environmental conditions that may initiate and be relevant to disease are the subject of this investigation. Despite extensive research, the mechanisms by which *S. enteritidis* causes disease are still not fully understood. While there have been recent advances in some aspects of pathogenesis, other areas, such as the recent dramatic increases in the predominance of particular serotypes remain unresolved (Lax *et al.* 1995).

One of the main reasons for investigating *S. enteritidis* PT4 is the dramatic increase in the incidence of infections due to this organism. The decade from 1980 witnessed a rise of over 170% in the number of reported cases of salmonellosis in England and Wales, primarily due to a dramatic increase in *S. enteritidis* PT4 infections, (Fig. 1.2, data from PHLS, CDSC). Since 1990 the number of human isolations of *S. enteritidis* PT4 in England and Wales, as reported to the PHLS, Laboratory of Enteric Pathogens and CDSC, has stabilised at around 14,000 cases per annum.

The incidence of other serotypes of *Salmonella* has remained relatively constant over this time. As the incidence of *S. enteritidis* PT4 has increased, so the proportion of cases due to other serotypes has fallen from approximately 50% to 18%, and *S. enteritidis* PT4 has overtaken *S. typhimurium* as the most common cause of salmonellosis (Advisory Committee on Microbiological Safety of Foods, 1993). Increases in the occurrence of *S. enteritidis* have also been observed in other countries, creating a current global pandemic (Rodrigue *et al.* 1990). Although this increase in incidence has primarily been associated with poultry (Section 1.5) there is little knowledge of the factors that have resulted in this pandemic, and why the predominating phage type varies in different global regions. In the UK, Europe, and some other areas of the world, PT 4 has become the predominant phage type causing human disease, whereas PT 8 and 13a are the most common in the USA.

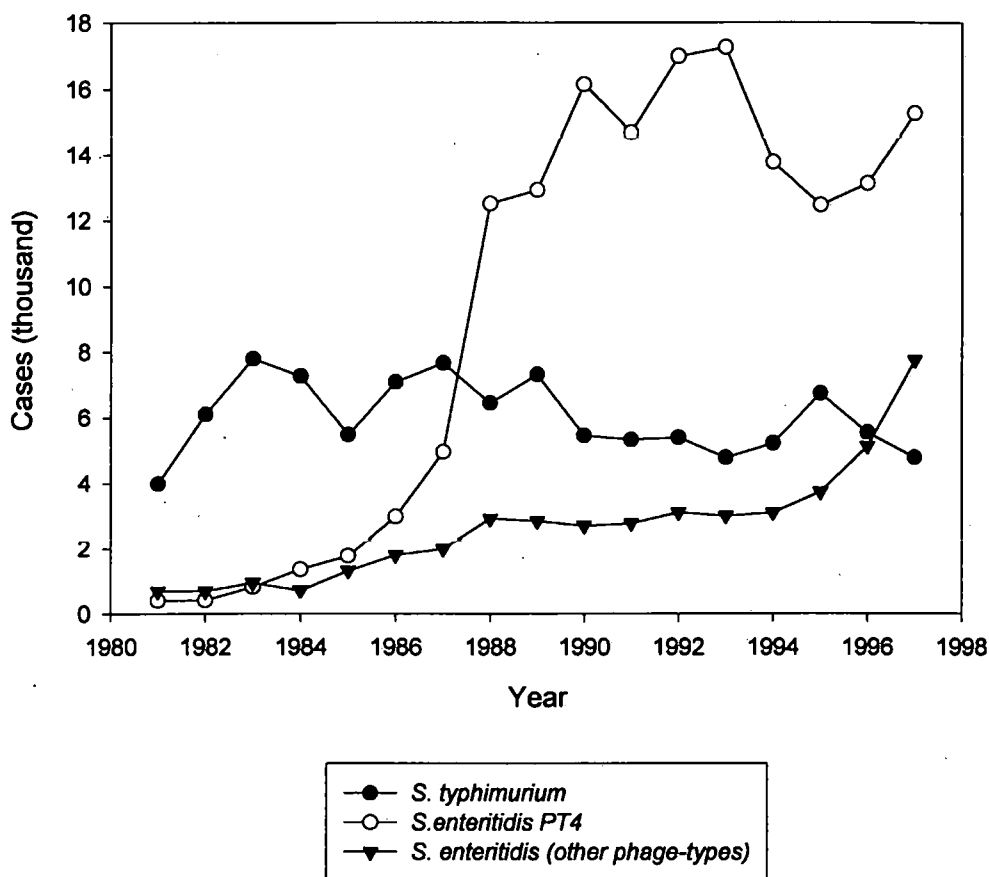


Figure 1.2 Reported cases of *Salmonella* infections in humans in England and Wales (PHLS, CDSC data)

The situation by 1990 was quite grave, particularly in poor countries that lacked sufficient resources or capital to eliminate the unsanitary conditions responsible for the transmission of the microbes. *Salmonella* became a problem not only in the Third World but also increased in incidence across the world, appearing in all locations from the Caesar salads served up in restaurants in Manhattan to street food stands in Poland (Karlen 1995).

It is estimated that sporadic, individual cases of salmonellosis account for about two thirds of all *Salmonella* infections (Roberts and Sockett 1994) and, consequently, many cases of infection due to *S. enteritidis* go unrecorded. Asymptomatic infections are also missed, making the incidence much greater

than suggested by recorded cases data. Studies in the United States have suggested that only between 1 in 29 and 1 in 145 cases of salmonellosis are recorded (Roberts *et al.* 1989). There are no substantiated data on the extent of under-reporting in the UK, although estimates of between 1 in 10 and 1 in 100 have been made (Anon. 1990). If the confirmed incidence is multiplied by this lowest estimate then the actual number of cases of *S. enteritidis* PT4 infection in England and Wales would be in the region of 150,000 per annum.

1.5 Association of *S. enteritidis* PT4 with poultry

Epidemiological investigations of foodborne salmonellosis have identified the primary source of *S. enteritidis* PT4 infection in the UK, to be hen eggs and, to a lesser extent, poultry meat (Coyle *et al.* 1988; Maloney and Guerrant 1992; Notermans and Hoogenboom-Verdegaal 1992). The incidence of *S. enteritidis* infection of poultry flocks has increased since 1986. *S. enteritidis* PT4 causes an invasive, asymptomatic infection in poultry that leads to septicaemia and subsequent chronic infection of various organs. If the ovaries become infected then transmission to eggs can occur. Consequently, *S. enteritidis* PT4 is found sometimes in the contents of eggs, (Gast and Beard, 1990; Suzuki, 1994; Humphrey, 1994) and within poultry meat muscle as well as contaminating the surface of these products (Wilson *et al.* 1996).

The frequency at which *Salmonella* contaminate the content of hen eggs varies; a level of 6 contaminated eggs per thousand from flocks known to be infected with *S. enteritidis* PT4 has been reported (Humphrey *et al.* 1989, 1991b). The frequency of egg shell contamination has been estimated to be higher, at about 1% (Humphrey *et al.* 1991b; Jones, F.T. *et al.* 1995).

No specific reason has been identified to account for the increased prevalence of *S. enteritidis* in poultry, although a number of suggestions can be postulated. For example, a point source outbreak may have occurred in an elite breeder stock. The initial vehicle of infection may have been feed, additives or a contaminated vaccine or probiotic. The use of nitrofurans in the poultry industry, which disrupts the commensal flora and subsequently may encourage the infection and colonisation of poultry by *S. enteritidis*, have also been implicated (Rampling *et*

al. 1990). Changes in flock susceptibility due to the production of hybrid strains could also influence the incidence of infection. The increased movement of livestock and food stuffs internationally has increased the potential for the global spread of infectious agents such as *S. enteritidis*. In England PT4 is the predominant serotype in broiler chickens and may be excluding other serotypes (Rampling *et al.* 1989).

About 50% of outbreaks where a food source has been identified are caused by poultry or poultry products (Advisory Committee on Microbiological Safety of Food, 1993). This initially caused the public to lose confidence in these products, particularly eggs. In 1988, the British government contributed £7 m to support the egg industry following reduced consumer demand for these foodstuffs after the disclosure of the link between increased *S. enteritidis* infection and contamination of poultry and eggs.

Changes in poultry production processes has increased the output of this industry. Poultry is making a significant contribution to the requirement for cheap, quality protein, and for a multitude of seasoning products. The consumption of poultry meat has increased as a result of outbreaks of bovine spongiform encephalopathy (BSE) in cattle and the consequential decline in the demand for beef products (Meslin 1992). Together with poultry meat and its derivatives, eggs have a vast consumer market both in their natural form and also incorporated into other products, (sometimes in a raw state). The increased consumer demand for these products emphasises the requirement to ensure products are of the highest microbiological standard.

1.6 Routes for transmission of *S. enteritidis*

Salmonellosis in animals and man is usually due to the consumption of contaminated food or water although secondary spread may occur directly via the faecal-oral route e.g. among intensively-farmed animals. Food of animal origin is regarded as the major source of *Salmonella* infections in humans. The faecal effluent from infected animals and man is an important source of contamination of the environment resulting in cross-contamination of food chains (Fig. 1.3) (Baird-Parker 1990).

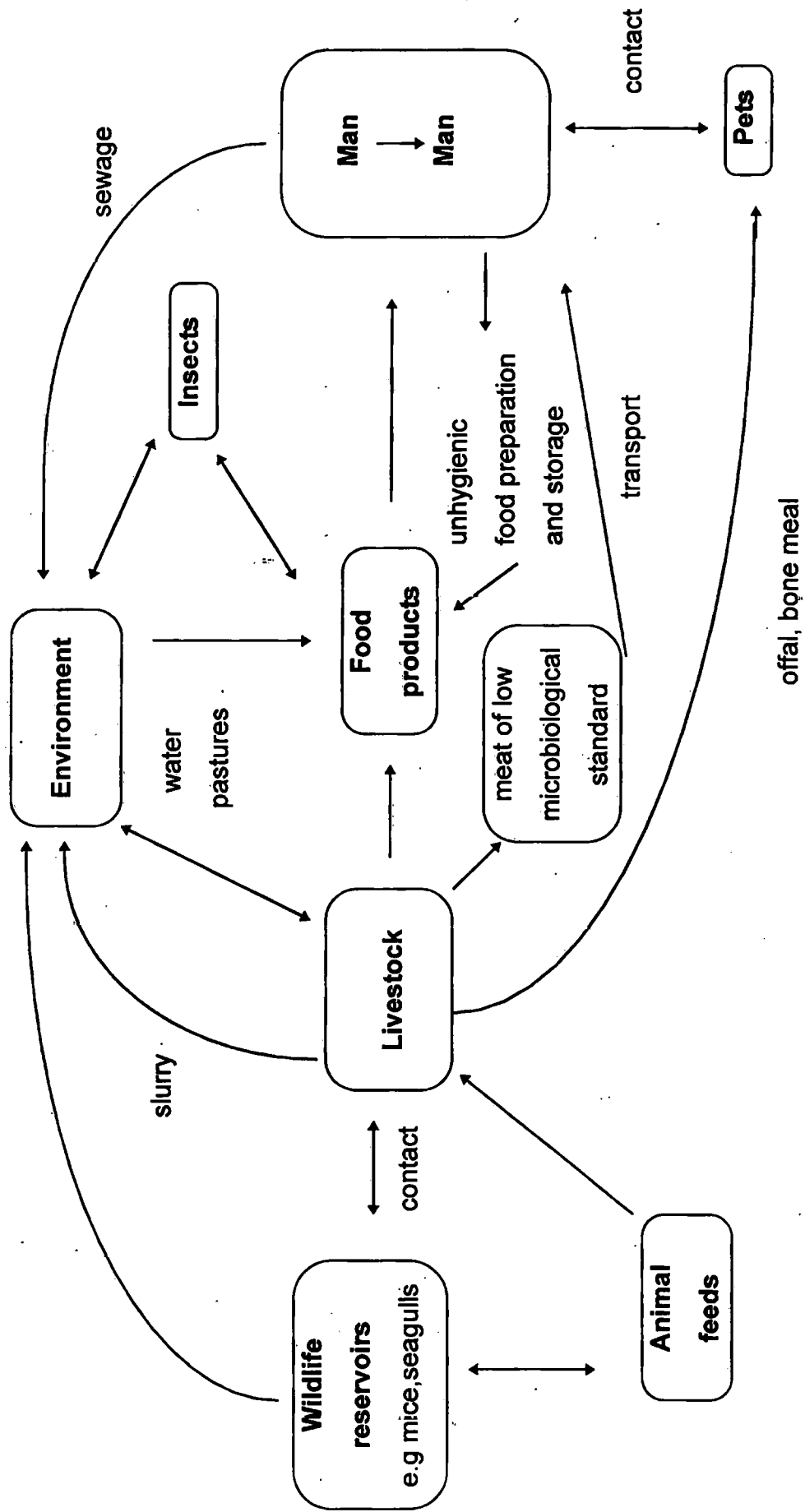


Figure 1.3 Routes of transmission for *S. enteritidis* PT4

It is commonly assumed that the primary source of foodborne disease is from commercial outlets such as hotels, restaurants, fast food establishments etc. as these receive the most publicity. However, in countries which routinely monitor outbreaks, (such as the UK), a high proportion (estimates of up to 80%) of outbreaks can be attributed to family households. As it is not usual to confirm if the source of infection was from within the home, there are no data available on the proportion of family outbreaks which are due to poor hygiene practices. Family outbreaks being defined as two or more cases within the same household.

The level of cross-contamination within the domestic kitchen has been shown to be high. Contamination of fingers, cooking utensils and work surfaces was observed following mixing of egg products. Mixing caused a large 'splatter zone' onto the work surfaces and it was observed that *S. enteritidis* PT4 could be isolated from the dried droplets 24 hours later (Humphrey *et al.* 1994). The ease with which surfaces in a kitchen can become contaminated both by direct contact with a contaminated food product or by transfer of bacteria on the hands is emphasised by the findings of a survey which indicated that even relatively brief contact of fingers or kitchen utensils with a contaminated surface transferred significant numbers of bacteria which could be recovered up to 24 hours later (Scott and Bloomfield 1990).

These data indicate the important role that indirect transmission may have in the domestic kitchen. Moist areas within the kitchen e.g. sinks and waste pipes are reservoirs for bacteria, and various species of bacteria have been found to survive on both soiled and clean dishcloths for long time periods (Scott and Bloomfield 1993). Although drying plays an important role in reducing the level of contaminating bacteria on hard and soft surfaces, it is not sufficient to prevent transmission of viable bacteria. Resistance to the lethal action of passive air drying significantly increases the potential spread of bacteria.

Illness within a family obviously increases the opportunity for cross-contamination within the home, with toilets and sinks becoming additional reservoirs for

pathogens, and towels, handles and surfaces becoming possible disseminators. Although the home has an important role in the spread of foodborne disease, little attention has been focused on education towards an effective cleaning /disinfection protocol for general use. This is an avenue which should be exploited, particularly for homes of susceptible individuals.

Outbreaks of *S. enteritidis* in hospitals, rest homes and other institutions are not uncommon and occasionally contribute to death. The new-born, elderly and those with diminished resistance to infection are most at risk. Poor sanitation in kitchens and wards increases the likelihood of exposure to *Salmonella* particularly in institutions. Once established, infections may pass from person to person for long periods of time. *Salmonella* carriage by catering staff can lead to disease, highlighting the importance of improving the awareness of catering staff (Hedberg *et al.* 1991; Dryden *et al.* 1994). Problems can be reduced by control of infection procedures being in place, these include appropriate training of all staff handling food and isolation of infected patients (Lightfoot, N.F. *et al.* 1990).

1.7 Increase of domestic salmonellosis due to exotic pets

Various countries throughout the world have associated cases of domestic salmonellosis with the handling of pet reptiles and turtles (Weil *et al.* 1995). The majority of the cases are children and the infection can be severe requiring hospital care. Ironically, many of the children adopted these pets to avoid allergic reactions to furry animals. The exportation of these infected animals is increasing the spread of organisms between countries. More information to prospective buyers of these pets and tighter controls on the exportation of carrier animals is required.

1.8 Spectrum of disease

In man salmonellosis is generally defined as the less serious type of gastro-enteritis transmitted by non-enteric salmonellae. Non-typhoid *Salmonella* infection can also result in bacteraemia, a chronic carrier state and localised infection (Ramos *et al.* 1996). Many salmonellae are host specific (**Section 1.3**), generally, the more host restricted the serotype the more likely it is to cause

systemic disease. *S. enteritidis* is not host specific and generally does not result in systemic disease.

Salmonellae multiply in the small intestines and colon after ingestion, leading to inflammation of the lamina propria. They may colonise the intestine without causing disease as close association and penetration of the intestinal mucosa are prerequisites for diarrhoea and systemic disease. If the bacteria are not constrained by the mucosal and lymphatic barriers, access to the bloodstream can occur; the resulting bacteraemia may be transient and inconsequential or may lead to metastatic infections and to persistent bacteraemia. The existence of over 2,000 serotypes of *Salmonella* suggests that naturally acquired immunity plays only a limited role in protection.

The principal symptoms of infection in humans are diarrhoea, abdominal pain, mild fever, chills nausea and vomiting (Baird-Parker 1990). The spectrum of illness caused by *S. enteritidis* in man ranges from mild gastrointestinal upset, which may not warrant seeking medical advice, to a more severe debilitating illness, resulting in dehydration, and infrequently, leads to death. The onset of clinical symptoms is usually 12 to 36 hours after infection. The incidence of bacteraemia due to *S. enteritidis*, in England and Wales is between 1-2% (Threlfall *et al.* 1992, 1998). Most infections in poultry are asymptomatic; *S. enteritidis* can cause chronic invasive infection of poultry flocks, sometimes causing a pericarditis resembling *S. pullorum* infection. In mice *S. enteritidis* causes a disease which is similar to human typhoid fever.

1.9 The socio-economic implications of *S. enteritidis*

The large number of *S. enteritidis* infections results in high economic and personal costs. In the UK these are estimated to run into millions of pounds annually for reported cases alone (Baird-Parker 1990). An evaluation of the impact of salmonellosis in England was made by studying 1,482 cases; the cost to the health care system, including case investigations, was estimated to be £400,000 while the cost to families was about £70 per case (Sockett and Roberts 1991). The costs pose an economic burden on both the public and private sectors. Economic costs can be attributed to:- monitoring and controlling the

spread of infection, and health care provision (predominantly for the more severe cases), loss of work time, and loss of reputation and customers for those producers/retailers associated with the contaminated product. The social costs include pain and discomfort to the individuals involved and disruption of their normal family life. A major portion of the costs reflect the loss in productivity with the average absence time from work estimated to be 6.5 days (Roberts and Sockett 1994).

Assuming that the economic costs incurred by an *S. enteritidis* infection are similar to those of other salmonellae, it has been estimated that the costs in 1992, due to *S. enteritidis* illness, in England and Wales was between £224 million and £321 million (Roberts and Sockett 1994). With the majority of the costs being associated with treatment, investigation and time lost from work.

The costs of long-term sequelae of *S. enteritidis* infection such as reactive arthritis (Hughes and Keat 1994) and the carrier state are still to be determined. The number of cases of chronic post-infection problems are estimated to be low, although the medical costs involved in their treatment may be significant.

The effect of *S. enteritidis* infections on the economy of developing countries has not been estimated as case records are not generally made, but it may be that the economic impact is even greater in these countries. The poor nutritional status of the population would be expected to predispose them to a more debilitating infection. Also, inadequate medical support and control measures could result in an increased number of fatalities.

1.10 Intervention strategies

There is no effective prophylactic procedure for the prevention of infection with *S. enteritidis* in man. As most cases of infection result from the ingestion of contaminated food, the best approach to prevention is to improve the microbiological standards of the frequently implicated food products. A high proportion of *S. enteritidis* infections may be prevented by intervention strategies to reduce the number of transmission routes into the human food chain. The economic costs of intervention strategies are likely to be large as they need to

target all areas of the food production chain (Fig. 1.3). Control at one point in the process would be compromised by less effective measures being employed at a later stage. The funds required for effective control are not known nor the effectiveness of different strategies, however, it could be assumed that the rate of return on effective control of *S. enteritidis* infection would be great due to the vast costs incurred by infections. Various preventative regimes are now in use in response to HM government advice, and the public have been made aware of the need to store and cook poultry and egg products in a suitable manner.

The obvious starting point for control of *S. enteritidis* is in poultry since a significant reduction in human infections would be anticipated if *S. enteritidis* could be excluded from poultry flocks. This is complicated as the poultry meat industry has a chain of production stages which include grandparent stock, elite flocks, breeder hatcheries, rearing parent stock, parent stock, egg collection and finally marketing which involves transport, slaughter and final product preparation and storage. Potential routes of transmission exist at all of the stages of poultry production, and contamination from the environment is significant. Indeed, open sided hen houses are common in countries with higher temperatures and the risk of contamination from environmental sources is great. Consequently, *S. enteritidis* eradication from the breeder flocks alone is not sufficient to exclude this organism from the poultry food chain as vertical contamination from external factors can occur at any stage.

Control measures such as vaccination (Timms *et al.* 1990; Cooper *et al.* 1992), or competitive exclusion flora, which involves young birds being exposed to normal non-pathogenic enteric bacteria which act competitively to prevent colonisation of the intestinal tract, have been shown to be useful in reducing the prevalence of *S. enteritidis* (Nurmi *et al.* 1991; Nuotio *et al.* 1992; Carrier *et al.* 1994).

Reducing the prevalence of *S. enteritidis* in the environment and in animal feeds is another method for controlling the incidence of *S. enteritidis*. This requires education of the personnel involved so that more hygienic policies can be

sustained at all production stages. Good infection control protocols must address:

- contamination from clothes and shoes, etc, of workers
- contamination from newly introduced birds
- adequate disinfection and cleaning of “risk” areas - avoiding aerosol formation
- the increased susceptibility to infection of stressed animals
- control of rodents, beetles, insects and birds
- safe disposal of hen house waste
- the provision of “safe” water, straw and foodstuffs
- monitoring and surveillance
- treatment / isolation of infected animals

1.11 Environmental survival of *S. enteritidis*

The various environments experienced by *S. enteritidis* during the transmission of infection present a multicomponent, complex series of physical and chemical stimuli. For *S. enteritidis* to be able to survive and grow under all the conditions persisting in the diverse environments from which it has been isolated the organism must be very adaptable and able to respond rapidly to deal with changes in the local environment. Implicated food products display a wide variety of environmental conditions; for example, the hen egg has several non-specific antibacterial systems. A few days after laying the pH of egg albumin is greater than pH 9, there is a high initial viscosity and many inhibitors such as transferrin, ovalbumin and lysozyme are present, and the concentration of available iron is very low (Board *et al.* 1994). **Table 1.1** illustrates the range of temperatures and pH values that may occur in some poultry products.

Table 1.1 Environmental conditions in some implicated foodstuffs

Food	pH	Storage / Cooking Temperature (°C)
Albumin	9	4, RT, 55
Mayonnaise	4	4, RT
Chicken meat	7	-20, 4, RT, 55

RT = room temperature

Clearly an infecting *Salmonella* cell will be subject to a succession of changing environments as it enters the digestive tract and passes through different regions of the gut. These include temperature shifts from ambient (food) to body temperature, transitions from acid (stomach) to alkaline pH (duodenum), the presence of bile salts and digestive enzymes in the small bowel and ultimately passage to the caecum and colon where competition with the commensal flora for nutrients results in a low nutrient, increasingly anaerobic environment with toxic bacterial products such as volatile fatty acids, bile acids and sulphide ions (Freter 1983). During systemic infection the bacteria also have to cope with various types of oxidative stress and a variety of antimicrobial peptides elicited by macrophages and lymphocytes (Foster and Spector 1995).

Despite many years of investigation, knowledge is incomplete on the ecology of salmonellae in the intestine and of the normal flora which may confer colonisation resistance. This is not surprising when one considers that there are 10 times more intestinal bacteria than host cells, and in man there are in excess of 10^{11} bacteria per gram of intestinal content (Mackie *et al.* 1997).

The interactions between host and pathogen during disease are dynamic. The bacterial strategies for survival and multiplication meet head on with the formidable defences of the immune system of the host and, to survive, the bacteria must respond rapidly to changes in their immediate environment (Mekalanos 1992).

1.12 Environmental adaptation

S. enteritidis must be able to detect changes in environmental factors and respond to protect itself against adverse conditions. Environmental conditions may vary rapidly such as occurs following the transfer from the oesophagus into the stomach; this means that the bacterial response must also be rapid. Methods of response include phase-variation, which controls the expression of cell surface components such as outer membrane proteins or adhesins, and is ideal for altering the structure of the cell when it is vulnerable to immune recognition. Protein phosphorylation and methylation are used in the transfer of physical and chemical information from the environment to the genome where it modulates the expression of relevant genes. Adaptation to dynamic environments requires the bacterium to modulate its transcriptional profile so that the appropriate genes are expressed as required. Changes induced by environmental modulation are classed as adaptation or stress responses. The phenotypic and genotypic changes induced by growth at different pH levels may influence subsequent responses to other stresses and ultimately pathogenicity.

The cellular stress response was first reported in 1962 for the heat-shock response of *Drosophila* (Ritossa 1962). Stress responses have been shown to protect organisms from exposure to a variety of adverse factors including high temperatures, high and low osmolarity, acid and base, as well as starvation. Stress responses involve the rapid synthesis of a range of proteins, which are termed stress proteins. Initially, the proteins were termed heat-shock proteins as they were first observed in response to heat stress. However, this is a misnomer, as a range of proteins are synthesised in response to many environmental factors, the mechanisms for some of which are distinct. Although these proteins have been termed "stress-proteins", and they do take on additional functions in repairing denatured proteins and protecting cellular proteins from environmentally-induced damage, they are not exclusively produced under hostile conditions, as some are produced to a certain degree under most environmental conditions. These proteins are highly conserved and have been observed in a range of organisms from bacteria to humans (Sanders 1993). Included in the stress proteins are a subset of proteins called molecular

chaperones which are responsible for the folding, assembly and translocation of other proteins (Langer *et al.* 1992) (**Section 6.1.10**).

Exposure to an initial hostile condition induces the production of stress proteins which enable the organism to survive when exposed to a more stringent challenge which would otherwise cause death (Lindquist 1986). Therefore, the accumulation of stress proteins correlates with an enhanced tolerance of extreme environmental conditions, which implies that these proteins must have an important ecological role. The acid response of *S. typhimurium* has been well documented by Foster and colleagues and has been shown to involve a series of complex responses which vary with the growth phase (Foster and Spector 1995) (**Section 4.1.4**).

Research into the stress responses of *S. enteritidis* is incomplete and the biological functions of the proteins induced during adaptation and stress are still to be identified. The main concerns are the potential roles for these proteins in the physiology, survival and virulence of cells as all these properties will influence the success of the organism as a pathogen. Understanding the regulation of virulence properties can help us define what constitutes a potential virulence factor and indeed facilitate the identification of new virulence factors on the basis of their regulatory properties. The expression of various virulence determinants is frequently co-ordinately controlled by a common regulatory system, which controls the expression of genes encoding these virulence determinants. For example in *S. typhimurium*, osmolarity, starvation-induced stress, pH and growth phase have been shown to be environmental signals controlling the expression of co-ordinately regulated virulence determinants. The molecular level of control is usually transcriptional, but more than one DNA-binding regulatory protein can be involved (Mekalanos 1992).

1.13 Virulence factors

Virulence as a concept is intrinsically coupled with colonisation and disease. Disease is most readily measured in terms of morbidity and mortality. However, the degree of host injury does not necessarily correlate with evolutionary success for a pathogenic microbe as the main priorities are persistence and multiplication.

Disease symptoms arise as a consequence of the dynamic, complex interactions which occur between the infecting pathogen and a host. Virulence determinants can be classified as factors which contribute to the persistence and multiplication of a pathogen *in vivo*, excluding 'house keeping' functions that are always required for replication irrespective of the environment. Some factors that have host-specific functions are easily classified as virulence determinants eg. adherence to host tissues, invasion into host cells and epithelia, and resistance to host defence mechanisms. Others have both virulence and 'housekeeping' functions dependent upon the external environment; for example, factors which facilitate the acquisition of iron are virulence factors for invading microbes, but, they are also required under iron-limiting growth environments *in vitro*.

The multifaceted, changing nature of the host-parasite interaction necessitates that more than one virulence determinant is involved in pathogenesis. Specific virulence determinants (e.g. adhesins, invasins, toxins, motility, etc.) contribute to unique steps in the pathobiology of microbes.

1.14 Why study adaptation to environmental pH?

pH was selected as the environmental factor for detailed study in this investigation due to its relevance to the persistence of *S. enteritidis* PT4 in:

- implicated food environments (*S. enteritidis* is frequently isolated in products with acid or alkaline pH values, e.g. mayonnaise and eggs, **Table 1.1**)
- different regions of the host encountered during infection (e.g. gastric barrier, phagolysosomes, macrophages)
- environmental reservoirs - acidic pH is one of the most frequent adverse conditions encountered in natural environments; alkaline pH levels tend to persist in natural waters which have been contaminated with industrial and agricultural pollutants.

The role of adaptation to different pH levels in virulence and tolerance is of great significance and will be discussed through-out this thesis.

1.15 Role of acid adaptation

Whether infection follows exposure to *S. enteritidis* depends partially on the number of bacteria ingested, but also on their physiological state. Large doses of

S. enteritidis are generally required to cause disease under experimental conditions, although epidemiological evidence suggests that the infective dose in humans can be quite small, (Blaser and Newman 1982). *S. enteritidis* gastroenteritis can be transmitted via the faecal-oral route, which again suggests that the minimal infective dose can be low.

Bacteria must be present in sufficient numbers to overcome local defences, to adhere to mucosal surfaces and to multiply before infection can be initiated (Smith 1995). Even extremely virulent strains may be cleared by host defences if the cell number is below the infective dose threshold (Blaser and Newman 1982). The dose of a pathogen required to cause infection can be measured both in terms of the frequency with which a specified dose causes infection or in terms of the time required for disease to become established (Blaser and Newman 1982).

An important determinant in the infective dose of ingested *S. enteritidis* cells could be previous acid adaptation. In the stomach, the bacteria are rapidly mixed with acid, at a pH below pH 3. Those already adapted to low pH in the food matrix, have a better chance of surviving in sufficient numbers to initiate disease. Whether any of the changes induced by acid adaptation in *S. enteritidis* have a role as virulence determinants *per se* (e.g. through enhanced attachment, and increased translocation etc.) is yet to be determined.

Acid adaptation has been shown to influence the tolerance of bacteria to hostile environmental factors. Pre-exposure of *S. enteritidis* PT4 to low pH *in vitro* has been shown to increase the tolerance of the bacteria to subsequent, and more stringent, challenges of low pH (Humphrey *et al.* 1993a). These mechanisms are significant in the food situation with acid adaptation increasing the survival of *S. typhimurium* in cheese (Leyer and Johnson 1992). Exposure of *E. coli* to alkaline conditions has been shown to increase its sensitivity to subsequent low pH challenge (Rowbury *et al.* 1993a). Adaptation of *S. enteritidis* PT4 to alkaline conditions also affected the thermo-tolerance of the bacteria (Humphrey *et al.* 1991a). An interdependency of some of the stress responses has been

observed with other environmental factors such as temperature and nutrient limitation influencing the acid tolerance of enterobacteria (Rowbury 1995).

1.16 Quorum sensing

The phenomenon of cell-density-dependent control of gene expression is called autoinduction (Quorum sensing). This has been of interest in bioluminescent marine bacteria for some time. It is now becoming clear that many other bacteria use an autoinduction mechanism to regulate a variety of cell functions. Cell-density-dependent gene expression is an example of multicellular behaviour by bacteria, where a minimum population size, i.e. a 'quorum', is required for certain functions of the population to be performed effectively.

N-acyl-homoserine lactones have been shown to be the autoinducers of luciferase synthesis in marine bacteria and also of pathogenesis functions in several Gram-negative pathogenic bacteria e.g. *E. coli* (Sitnikov *et al.* 1996) and *Pseudomonas aeruginosa* (Latifi *et al.* 1995; Winson *et al.* 1995). There may be a role for quorum sensing in the adaptation responses of bacteria. Environmentally stimulated release of extracellular signalling molecules by one cell may modulate the growth and gene expression of neighbouring bacteria.

1.17 Future outlook

Increasing numbers of immunocompromised patients, including those with AIDS, malignancies, renal transplants, etc., are contributing to a changing epidemiology of salmonellosis, as these groups have an increased incidence of bacteraemia. Other groups which have been identified as having an increased risk for salmonellosis are the elderly, the very young, those receiving acid blocking treatments and insulin-dependent diabetics; the postulated mechanisms for this observed increase include decreased gastric acidity and possibly reduced intestinal motility (Telzak *et al.* 1991).

In the human gastrointestinal tract, the dense population of commensal and potentially pathogenic microbes provide an ideal environment for events involving pooling of genetic information by transfection and plasmid exchange. Treatment of infections with antibiotics has encouraged bacteria to adapt and acquire

resistance, and as many of the required genes can be shared, this information has spread between microbial species.

Soon after the medical antibiotic revolution, an equally radical change in veterinary and livestock practices occurred. Expensive livestock with ailments could be treated with antibiotics. Prophylactic treatment became fashionable and included routine antibiotic administration to chickens, cattle and dairy cows. This had the added benefit of increasing feed conversion and gave rise to the use of low levels of antibiotics as 'growth promoters'. The shelf life of meat, poultry, eggs and dairy products was extended by previous antibiotic treatment of the animals. This practise meant that, in addition to humans taking courses of antibiotics, large numbers of animals were undergoing prophylactic exposure to or treatment with antibiotics, more than doubling the selective pressure on bacterial populations.

As early as 1970, it was shown that giving antibiotics to chickens resulted in the emergence of resistant *Salmonella* which could be isolated from both the meat and the eggs of the animals (Levy *et al.* 1976). Only thorough cooking could safely destroy the mutant organisms.

The majority of strains of *S. enteritidis* in the UK continue to be fully sensitive to antimicrobial drugs. For example, of 18,968 human isolates reported by the PHLS, Laboratory for Enteric Pathogens in 1996 only 8% were drug resistant, with less than 0.5% being multi-resistant, (resistant to four or more drugs). This is in contrast to observations with *S. typhimurium*, where 81% of human isolates in 1996 were multi-resistant, predominantly due to a strain of definitive type 104, (DT104). This gives rise to two major concerns, firstly the emergence of multi-resistant *S. typhimurium*, but also the fear of *S. enteritidis* acquiring similar drug resistance. Such a situation would be very serious due to the high incidence of *S. enteritidis* (Wall *et al.* 1994). Unlike *S. enteritidis* PT4, which has its reservoir in poultry, *S. typhimurium* DT104 is primarily a bovine pathogen but has spread to many animals and poultry. Therefore, a wide range of food stuffs may potentially become contaminated and the transfer of genetic information

becomes possible when *S. enteritidis* and *S. typhimurium* reside in the same habitats.

In 1983, an outbreak of *Salmonella newport* food poisoning across the USA illustrated a disturbing new trend. The causative agent was resistant to penicillin, ampicillin, carbenicillin and tetracycline. The patients were far sicker than usual, with six requiring hospitalisation for more than one week. The implicated food source was contaminated beef from a herd that had been given antibiotics at levels below the legal standards. Antibiotic resistance had developed in the *Salmonella* that were infecting the cattle. Most of the humans demonstrating the more severe symptoms were taking antibiotics when they consumed the contaminated meat. The first antibiotics had cleared many commensal organisms in the humans, creating a less competitive environment for *S. newport* colonisation. Despite this, and a host of other examples of the transmission of antibiotic resistant bacteria from meat, dairy and poultry products to human consumers, the U.S. Food and Drug Administration, its counterparts in the European Community, have taken only limited action to reduce the use of antibiotics on animals. Government agencies are reluctant to take steps which might impinge upon their country's competitive status in world agricultural markets (Garrett 1995).

Although the use of antimicrobials in poultry has not been as extensive as in cattle, there is increasing evidence that their use in poultry is contributing to the emergence of strains with decreased sensitivity to these compounds (Rampling *et al.* 1990; Frost *et al.* 1996).

It is still unclear whether the increase in the incidence of *S. enteritidis* PT4 in human salmonellosis is caused solely by its increased prevalence in poultry. Any one or a combination of the following could be the cause:-

1. an increase in the virulence of the organism for man
2. entry into a widely consumed food source
3. increased consumption of inadequately pre-prepared/heated food products
4. an increased ability to survive extreme environmental conditions

5. disruption of the poultry commensal flora by antibiotic-laced feeds allowing infection and colonisation by *S. enteritidis*
6. an ability to out grow competing organisms
7. poor farm hygiene practices and large flock sizes
8. inadequate food handling hygiene policies
9. poor slaughter house practices which allow cross-contamination of products.

1.18 Aims

The starting point of the investigation was to identify indicators as to why *S. enteritidis* and in particular PT4 have caused a pandemic of salmonellosis since the mid 1980's. The initial aims were, therefore, to compare:-

- the ability of *S. enteritidis* PT4 to grow under a range of extreme conditions relevant to food and host environments, with that of other serotypes and phage types of *Salmonella*.
- the growth rates of isolates of *S. enteritidis* PT4 with those of other serotypes and phage types of *Salmonella* under standardised conditions.

Following on from these initial studies, the influence of growth at different environmental pH levels on a clinical isolate of *S. enteritidis* PT4, (strain ref. 226 405) will be determined. A chemostat system will be used to grow cultures under defined conditions at different pH values (acid, neutral and alkaline). These cultures will be used to investigate:-

- changes in the expression of surface structures and plasmid carriage in response to growth at extremes of pH
- the cross-protection provided by pH-adaptation to other environmental stress factors
- the relationship between pH adaptation and pathogenicity in a mouse model
- changes in protein and phospholipid expression in response to growth pH
- the characteristics of some of the proteins involved in the pH stress responses of *S. enteritidis* PT4.

The results from these investigations will be reviewed with the following questions in mind:-

1. Does *S. enteritidis* PT4 have inherent properties which confer a selective advantage when compared to other serotypes and phage types of *Salmonella*?
2. What changes occur in *S. enteritidis* PT4 when grown at extremes of pH?
3. Is there a role for the pH adaptation response(s) in the pathogenicity of *S. enteritidis* PT4?
4. Are stress proteins involved in the adaptation strategies of *S. enteritidis* PT4, to growth at extremes of pH?

Chapter 2

A Comparison of the Growth and Tolerance of a Variety of Serotypes and Phage Types of *Salmonella* to a Range of Extreme Environmental Conditions

2.1 Introduction

Illnesses due to food contaminated with either chemical or biological agents, are one of the most widespread problems throughout the world. Comparison of data between countries and the determination of actual figures is confounded by under reporting. Frequently, patients do not seek medical advice, and also many countries do not record individual cases of illness (Notermans and Hoogenboom-Verdegaal 1992). However, there has been a significant increase in the number of reported cases of *S. enteritidis*-associated food poisoning, around the world, since the mid 1980's (Rodrigue *et al.* 1990). The implicated isolates are not always of the same strain and the predominating phage type varies in different regions of the world, for example *S. enteritidis* PT4 is the most prevalent in England and Wales (Fig. 1.2), whilst PT 8 and PT13a predominate in the USA (Schroeter *et al.* 1994). There is little knowledge of the factors that have contributed to this increased prevalence of *S. enteritidis*, nor why the strain and predominant phage type varies regionally, although it is logical to assume that it is multifactorial (Section 1.17).

2.1.1 The contribution of poultry products to the incidence of *S. enteritidis*

There has been an increased frequency of *S. enteritidis* PT4 carriage by poultry since 1986, the reasons for which are still unclear. *S. enteritidis* PT4 has been isolated from the muscle, skin, egg shell and egg content of infected hens, demonstrating that this organism is capable of reducing the microbiological quality of all poultry food products (Humphrey *et al.* 1989; Rampling *et al.* 1989; Humphrey 1990a). The consumption of poultry products has increased

significantly in recent years, and with more intensive farming techniques, chicken and eggs have become an economic source of protein.

2.1.2 The contribution of inadequate preparation and storage of food products to the incidence of *S. enteritidis*

Food poisoning outbreaks due to *S. enteritidis* PT4 have been traced to a variety of products in varying establishments and domestic residencies around the world. Many sporadic cases result from inadequate appreciation of hygiene standards and poor domestic practises (Ryan *et al.* 1996). The eating habits of the population have also changed considerably in the last decade, with the consumption of pre-prepared foods increasing dramatically. Processed foods are prepared on a large scale and then stored for long periods in chill-cabinets within the retail outlet. Many products e.g. sandwiches containing salads, eggs and mayonnaise, are at risk of serious contamination if the raw materials do not have a high microbiological standard and/or the storage conditions do not restrict bacterial replication. These products are frequently associated with outbreaks of disease (Cowden *et al.* 1989). Bacteria which can replicate or even survive at low temperatures can become an increased proportion of the contaminating flora in chill-stored products. If the food preservative factors become compromised there is a greater chance of pathogenic bacteria achieving/maintaining an infective dose level.

2.1.3 Pathogenic bacteria

To be a successful pathogen a bacterium must be highly adapted, being able to detect and respond to changing environments. A pathogen must be able to endure a range of environments which will provide access to a host. Once within the host, bacteria must be able to overcome the different anti-microbial factors which constitute the host defence mechanisms (Finlay and Falkow 1989). Pathogens must also be able to out-compete other microbes to enable their own proliferation and colonisation. An enhanced resistance to hostile conditions would confer a selective advantage to a bacterium. Bacterial populations are frequently in a state of continuous dynamic equilibrium; changes in the environment can perturb the proportions of the constituent bacterial members. The flora changes will reflect the ability of the strains to compete under the

prevailing conditions, with the more tolerant strains dominating. As more resistant bacteria are better able to increase or maintain their numbers over long time periods in fluctuating environments, the chances of their numbers exceeding the infective dose when a host is encountered are increased.

2.1.4 Environmental factors

Most environmental conditions have an interactive effect on the growth and survival of bacteria (**Fig. 2.1**). Shortage of any essential nutritional requirement, or extreme physical conditions can restrict the growth of bacteria, (both rate and yield), and these effects, can be bacteriostatic or even bacteriocidal. Some environmental conditions can be modulated by the activity of the bacteria themselves.

Many environmental factors are interrelated, with changes in one factor inducing related changes in another parameter e.g. accumulation of bacterial fermentation products such as volatile fatty acids decreases the pH which in turn affects the Eh of the environment.

2.1.5 Food and host environmental factors

There are three main sites in which salmonellae must survive and compete if they are to establish disease in humans:-

1. within the raw food/drink product e.g. within the food animal and its immediate surroundings, including the air and the water supply (**Fig. 1.3**),
2. during processing, preparation and storage of the food product (**Table 1.1**),
3. within the human body; (**Section 1.11**).

Environmental factors which affect the survival of strains of *Salmonella* at these sites are discussed below:

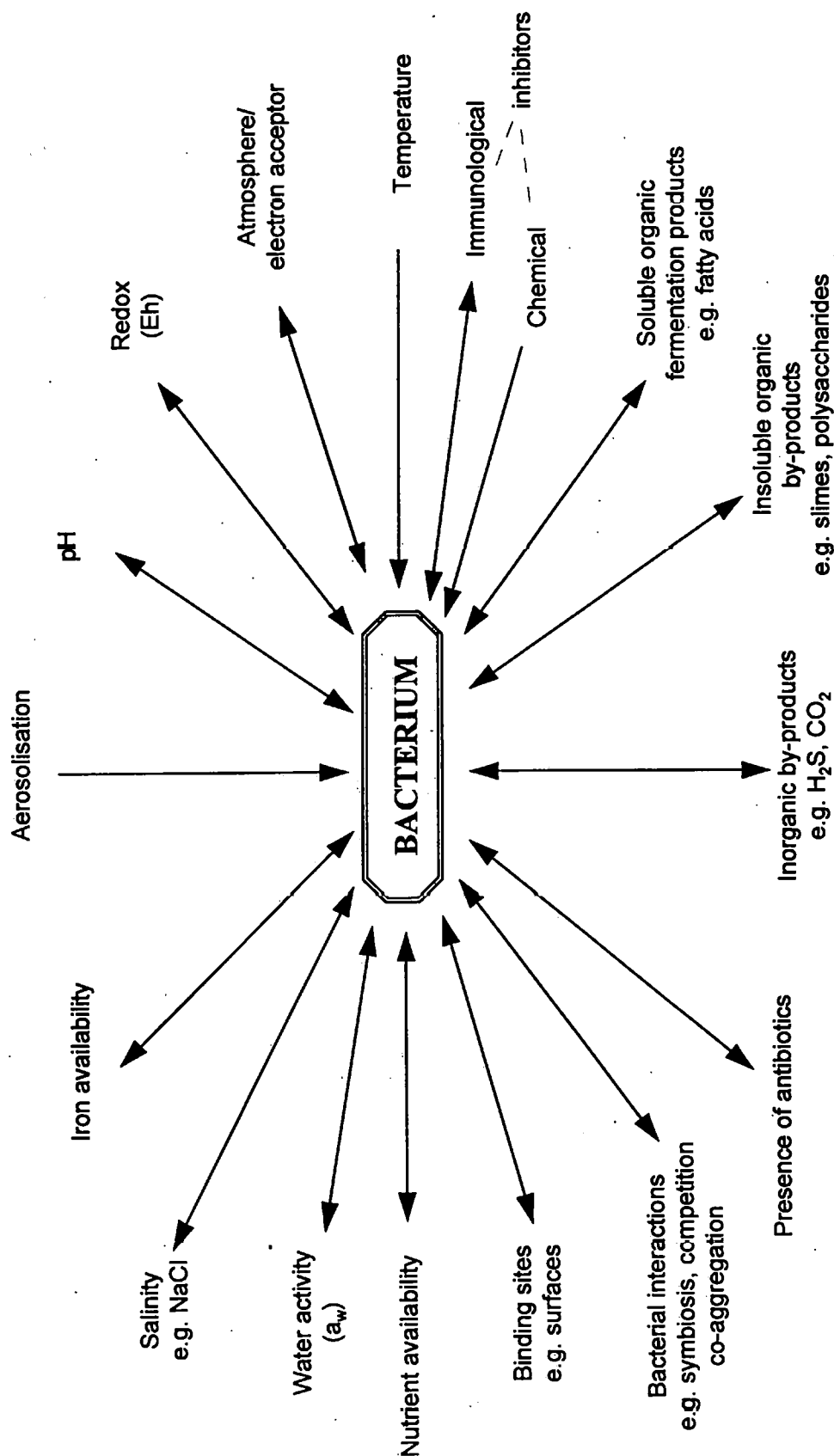


Figure 2.1 Environmental factors affecting bacterial replication and survival

2.1.5.1 pH

Outbreaks of food poisoning due to *S. enteritidis* PT4 have been associated with products with wide-ranging pH values e.g. mayonnaise, which has a pH value in the region of pH 4.0 (Smittle 1977), and egg albumin, the pH of which can reach pH 9.5 (Board *et al.* 1994). Hostile pH will also be experienced by an organism infecting the human body e.g. acid conditions will be experienced within the stomach (Giannella *et al.* 1972; Howden and Hunt 1987) and in phagolysosomes.

2.1.5.2 Temperature

The prevailing temperatures within food products will vary over large ranges during preparation, cooking and storage (**Table 1.1**). Smaller variations are observed in the temperatures of host species which salmonellae colonisation, for example 42° C in the hen and 37° C in humans, although salmonellae do infect reptiles with lower temperatures (**Section 1.7**).

2.1.5.3 Water activity (a_w)

The water activity (a_w) of a food product can be described as the amount of water available for microbial growth and determines the likelihood of bacterial proliferation (Mossel *et al.* 1995). Methods of food preservation which involve reducing the water activity of a product have long been recognised, and include drying and smoking, which directly remove water, and also salt curing and candying with sugar where the solute concentration is increased.

The relative humidity of the air in the storage of raw meats and food products also controls the growth of bacteria on the surface of these products (Baker 1990). The relative humidity will also affect the survival of bacteria in aerosols and their potential for respiratory infection and cross-contamination.

2.1.5.4 Resistance to the bactericidal activity of serum and lysozyme

When bacteria enter a host animal, an array of specific and non-specific chemical and cellular defences are encountered whose role is to eliminate the bacteria. A successful pathogen must be able to avoid, tolerate or inactivate these mechanisms in order to initiate disease. One mechanism depends on the surface

molecules of the bacterium being resistant to complement-mediated cell lysis, termed serum resistance (Patrick and Larkin 1995). Many Gram-negative bacteria are susceptible to the bactericidal activity of serum; however, strains demonstrating serum resistance are frequently isolated as causative agents of infections (Taylor 1975; Olling 1978). Resistance to serum activity has been shown to be an important virulence factor in Gram-negative bacteria during extra-intestinal infection (Leying *et al.* 1990).

Serum killing is frequently, but not invariably, accompanied by bacteriolysis which requires the presence of sufficient quantities of host lysozyme. Lysozyme hydrolyses the peptidoglycans of bacterial cell walls and is a naturally occurring antimicrobial factor present in egg albumin, (Tranter and Board 1984). It is produced by some bacteria as an extracellular enzyme and also has an important role in protection against infection in humans.

2.1.6 Growth rate as a factor in bacterial competition

An ability to replicate at a faster rate under diverse environmental conditions is an obvious method for out-competing other bacteria. This study includes a comparison of the growth rates of a range of *Salmonella* serotypes in minimal medium supplemented with mucin. A minimal medium was selected since many natural environments are hostile and do not support unrestricted bacterial replication. Mucin, was included as a complex source of nitrogen and carbon, as most bacterial infections occur on surfaces in the host in the presence of glycoproteins such as mucin (Smith 1995). The medium was designed to compare the growth rates of different bacteria under sub-optimal conditions, and not to simulate a specific natural environment.

2.1.7 Cell surface hydrophobicity and motility

Information concerning hydrophobicity and motility would indicate if some of the surface properties of *S. enteritidis* confer a selective advantage when compared with other salmonellae. Bacterial cell surface hydrophobicity has been shown to be a virulence factor for some pathogens (Siegfried *et al.* 1994). The hydrophobicity of the surface governs the attachment properties of the cells. Motility is also associated with pathogenicity; colonisation of the bladder by

S. typhimurium during experimental infection of the urinary tract of mice has been found to depend on motility (Siitonen and Nurminen 1992). Intact motility also enhanced the ability of *S. typhimurium* to enter cultured human colonic epithelial Caco-2 cells (Betts and Finlay 1992). Motility has a key role in chemotaxis improving the survival chances of an organism (Manson 1992). Flagella were not expressed when *S. enteritidis* PT4 was grown in the peritoneal cavity of chickens (Chart *et al.* 1993), however, and this suggests that flagella may have a specific function related to disease processes which occur early in the course of infection in the gastrointestinal tract.

2.1.8 Survival in aerosols

Although *Salmonella* infection is primarily via the foodborne or faecal-oral routes, respiratory infection is possible (Tannock and Smith 1971). *S. enteritidis* can be carried on dust particles or in airborne droplets, and previous studies have shown that systemic infection can occur in chickens challenged with a low infective dose via the respiratory and conjunctival routes (Baskerville *et al.* 1992; Humphrey *et al.* 1992). Successful transmission via the aerosol route requires that a number of criteria are met, such as a resistance by the organism to atmospheric exposure and drying.

This study was undertaken to assess the ability of *S. enteritidis* PT4 and *S. typhimurium* to remain viable in significant numbers when aerosolised under standardised conditions, and to relate this to their potential infectivity via the aerosol route. Many serotypes of *Salmonella* are capable of causing gastro-enteritis in humans. However, due to the time-constraints, it was not possible to study all serotypes. *S. enteritidis* PT4 and *S. typhimurium* were chosen for study as these are numerically the most significant causative agents of human food poisoning (Advisory Committee on Microbiological Safety of Foods, 1993). The purpose of this study was to compare the ability of a clinical isolate of *S. enteritidis* PT4 and of *S. typhimurium* to survive within aerosols, and to relate this to natural transmission thought to occur in poultry stocks.

In man, the airway epithelium has a large surface area, approximately 70 m², which is exposed to the whole volume of air we breathe (10,000 - 20,000 l.day⁻¹),

along with the dust and micro-organisms it contains. The air is cleaned, warmed and humidified during inspiration. A series of defence mechanisms are encountered during passage through the large airways, (nose, trachea and bronchi) where; for example ciliated epithelium lines the tracheobronchial tree down to the level of the terminal bronchioles trapping particles and preventing penetration to more vulnerable sites. Secretory cells line these airways and produce a number of substances with antibacterial activity, namely, lysozyme, lactoferrin (a glycoprotein which chelates iron), IgA and mucus glycoproteins which bind some bacterial particles (Richardson 1988). Some viable micro-organisms do reach the vulnerable alveolar surface and can then initiate infection.

2.1.9 Aims

As stated above, a wide range of conditions prevail in the food products implicated in outbreaks of disease with *S. enteritidis* PT4 (**Section 1.11**). This indicates that *S. enteritidis* PT4 must be able to tolerate a diverse array of hostile conditions. The aim of this investigation was to identify properties of *S. enteritidis*, and in particular of PT4, which may have contributed to its increased prevalence in poultry and human disease. Enhanced persistence, transmission or ability to out-compete other bacteria could all contribute to an increased predominance of an organism.

The overall aims of this initial investigation, therefore were to:

1. compare the growth rate, under defined conditions, of a range of *Salmonella* strains,
2. determine whether representative strains of *S. enteritidis* PT4 differed in their ability to grow under adverse conditions (relevant to food and host environments) when compared to other serotypes and phage types of *Salmonella*,
3. compare some inherent properties of *Salmonella* strains, e.g. motility, hydrophobicity, resistance to lysozyme activity,
4. identify the role aerosol survival may have in the persistence and transmission of *S. enteritidis* PT4 and *S. typhimurium*.

2.2 Methods

2.2.1 Bacterial strains

A selection of *Salmonella* strains were included in the study and are described below:-

- a range of clinical and type strains of *S. enteritidis* PT 4 - to provide information on the inherent variation within this group,
- other phage types of *S. enteritidis* (PT8 and PT13a),
- isolates of the avian-adapted species *S. pullorum* and *S. gallinarum* (the causative agents of fowl typhoid in poultry (Barrow *et al.* 1994)); extremely high doses of these serotypes are required to initiate disease in humans,
- *S. typhimurium* - one of the most commonly-isolated serotypes from human and animal diseases,
- *S. choleraesuis* - host adapted to pigs,
- *S. virchow* - the human incidence of which has recently increased.

The strains, and their origin, are listed in Table 2.3.

The survival of clinical isolates *S. enteritidis* PT4 (strain 226 405) and *S. typhimurium* (strain Swindon) in aerosols was determined; these were isolated and kindly provided by PHL, Salisbury, Wiltshire, UK, and P.A Barrow, Institute for Animal Health, Compton, Berkshire, UK, respectively.

2.2.2 Bacterial tolerance

The level of each challenge factor (listed in the following sections) was increased until a discriminatory value was obtained. The ability of the strains to grow at this discriminatory level was then determined; a weak result was recorded when only limited growth occurred.

2.2.2.1 Tolerance to humectants

Bacterial tolerance to high concentrations of sugar or salt was determined by growth on nutrient agar plates (NA), (bioMerieux) supplemented with 7.5% w/v sodium chloride, 50% w/v sucrose or 40% w/v glucose. Bacteria were plated onto the surface of the agar and incubated at 37°C in air for 48 hours, prior to recording the presence or absence of growth.

2.2.2.2 Tolerance to ethanol

5% v/v ethanol was included in full bottles of nutrient broth (NB), (Lab M, Wash Lane, Bury, UK.); these were inoculated with a loopful of the test strain and then incubated at 37°C for 96 hours with the caps sealed to eliminate evaporation. The presence of bacterial growth was determined visually and confirmed as *Salmonella* by subsequent plating onto a selective medium, (XLD, bioMerieux).

2.2.2.3 Tolerance to extremes of pH and temperature

Temperature tolerance was determined as the ability of bacteria to grow when spread on NA plates incubated at 5°C and 48°C in air for 96 hours. Tolerance to pH was assessed by adjusting the pH of the nutrient agar to pH 4 or pH 10 using 2N HCl or 2M NaOH, prior to pouring into plates; the pH of the plates was confirmed using a surface pH electrode (Mettler Toledo). The plates were incubated at 37°C in air for 48 hours prior to recording growth.

2.2.2.4 Serum resistance

The method used was based on those previously published (Olling 1978; Bengé 1988); in brief, an overnight culture was diluted in pre-warmed saline, (to exclude any 'heat shock' involvement), to approximately 10^4 cfu.ml⁻¹. 0.2ml of this culture was mixed with 0.2ml of male human serum (Sigma, Poole: prod. No. H1388) and incubated, with shaking, at 37°C. Samples were removed at time intervals and serially diluted using Maximum Recovery Medium (MRM), (Unipath) prior to spreading onto blood agar plates for estimations of viable cell number. The number of bacteria surviving was expressed as a percentage of the initial cell number; bacteria were recorded as resistant, (R) where the percentage survival was equal to or exceeded 100%, and as sensitive, (S) when the percentage survival was less than 100%.

2.2.2.5 Resistance to lysozyme activity

Bacteria were grown in batches of nutrient broth at 37°C in air for 48h, at which time the cultures were assumed to have reached stationary phase. 100µl of lysozyme solution: (23mg of lysozyme containing 48,000 units mg⁻¹ solid, was dissolved in 100ml of 0.067M phosphate buffer, pH 7.5 [lysozyme was from chicken egg white; Sigma product reference L-6876]) was added to 1.5ml of

bacterial culture. The mixture was incubated at 37°C in a cuvette and the rate of decrease in absorbance at 450 nm was recorded over 20 mins against a water blank (Barman 1974). Viable counts were also performed at the beginning and at the end of the experiment. Loss of cell integrity was determined using both optical density and viable count measurements. Organisms were recorded as resistant (R) when the cell number increased during the test, sensitive (S) when the viable cell number declined, and the effect was bacteriostatic (B) when the count remained unchanged.

2.2.3 Comparison of some Inherent properties of the test strains

2.2.3.1 Growth rate determinations

Growth rates were determined in Vogel Bonner medium, (Vogel and Bonner 1956) supplemented with 0.25% w/v partially purified porcine stomach mucin (Sigma) (Glenister *et al.* 1988) and vitamins (Carlsson 1970) (VBM). The composition of VBM is shown in Table 2.1.

Table 2.1 Composition of modified Vogel Bonner growth medium

Constituent	Concentration (g per litre of distilled water)
MgSO ₄ .7H ₂ O	0.2
C ₆ H ₈ O ₇ (Citric Acid)	2.0
K ₂ HPO ₄ anhydrous	10
NaNH ₄ (HPO ₄).4H ₂ O (Sodium ammonium hydrogen phosphate)	3.5
Mucin	2.5

The pH of the medium was adjusted to pH 7.0, then sterilised by autoclaving at 15 pounds per square inch of steam (121°C) for 30 minutes. The vitamin solution was prepared separately (Table 2.2) and sterilised by passage through a 0.22µm filter (Sartorius, Germany); 20ml of the vitamin solution was added per litre of VBM immediately prior to use.

Table 2.2 Composition of the vitamin supplement

Constituent	Concentration (g per litre of distilled water)
Pantothenic acid Ca salt	0.1
Biotin	0.1
Thiamin HCl	0.1
Riboflavin	0.1
Nicotinic Acid	0.1
Folic Acid	0.1

20ml volumes of VBM equilibrated to 37°C were inoculated with 200µl of test organisms from an overnight culture grown in the same medium. The cultures were grown, with shaking, at 37°C in air; at time intervals, samples of the culture were removed and serially diluted in Maximum Recovery Medium (MRM), (Unipath prod. No. CM733) (Patterson and Cassells 1963). Aliquots of the dilutions were spread onto blood agar (BA) plates (bioMerieux UK Ltd, Basingstoke), which were incubated in air at 37°C for 48 hours prior to the estimation of colony forming units. Plots of log₁₀ N (where N = cell number) against elapsed time were made and the doubling time calculated from the regression equation of the exponential region of the graph. Exponential growth can be described mathematically by the following equation:-

the number of divisions per unit time = $\frac{\log N_t - \log N_0}{t \cdot \log 2}$

where N₀ = the cell number at time 0 and N_t = the cell number at time t

therefore the number of divisions per unit time = k = $\frac{\text{gradient}}{0.693}$

so that the doubling time (t_d) = 1/k = $\frac{0.693}{\text{gradient}}$

2.2.3.2 Motility estimations

Motility was assessed using sloppy agar (0.3% w/v) by the 'Craigie tube' method. The test organisms were grown on nutrient agar plates for 48 hours; a colony was then removed and spread over the agar within the central tube. The tubes were incubated at 37°C for 48 hours; motile organisms migrated down through the agar and outside of the central tube (Craigie 1931).

2.2.3.3 Cell surface hydrophobicity

The relative hydrophobicity of the serotypes was determined using the 'salting out' technique (Lindahl *et al.* 1981). The minimum concentration of ammonium sulphate which caused the cells to agglutinate when rocked on a microscope slide at room temperature for 2 min was determined, by mixing 200µl from an overnight culture (grown at 37°C in nutrient broth under air) with 200µl of ammonium sulphate solution. Hydrophobic bacteria tend to aggregate at lower salt concentrations than do hydrophilic strains. A positive result for agglutination was scored when obvious agglutination was present and clearing of the background culture occurred; a weak value was recorded when agglutination required between 2 and 5 min for the reaction to be completed. The minimum salt concentration yielding visible aggregation was recorded.

2.2.3.4 Growth under an anaerobic atmosphere

Blood agar plates were pre-reduced in an anaerobic cabinet for 48 hours prior to use. These plates were then streaked with the test bacteria and incubated for 72 hours within the cabinet under an atmosphere of 10% CO₂:10% H₂:80% N₂ at 37°C; visual bacterial growth was then recorded.

2.2.4 Survival in aerosols

The ability of *S. enteritidis* PT4 strain 226 405 and *S. typhimurium* Swindon to survive in aerosols was compared. *S. enteritidis* PT4 and *S. typhimurium* were selected as these are the most prevalent serotypes causing human infection in the UK.

2.2.4.1 Preparation of bacteria for aerosolisation

Plate- and broth-grown cells were compared for their ability to survive within aerosols. Plate-grown cells were grown overnight on nutrient agar plates at 37°C

in air; subsequently, colonies were scraped off into 10ml volumes of saline at 37°C, to give a cell yield of ca. 10^{10} viable cells ml^{-1} . This count was confirmed by performing serial dilutions of the culture using Maximum Recovery Medium and then spreading aliquots of the dilutions on to blood agar plates; colony forming units were counted after incubation for 48 hours in air at 37°C.

To obtain exponential phase cultures, 100ml volumes of nutrient broth (NB) at pH 7 were inoculated with 10ml samples from an overnight culture and then incubated at 37°C under air for 2.5 hours. The incubation time required for the cultures to enter exponential replication was previously determined by following the entire growth curves for the two test strains under these conditions; by performing viable counts at 20 minute intervals. When the broth-grown cells were in exponential growth a sample was removed and concentrated by centrifugation (10 min at 3,000g), and the cell pellet resuspended in saline at 37°C to give a yield of ca. 10^{10} viable cells ml^{-1} .

2.2.4.2 Aerosolisation of the bacteria

Aerosols of plate or broth grown cultures, prepared in saline as described above, were generated using a Collison three jet nebulizer (Hambleton *et al.* 1983) in conjunction with a Henderson apparatus (Druett 1969) and maintained within a rotating drum with a capacity of 55 litres, at a mean room temperature of 24°C and a mean relative humidity of 74% (Goldberg *et al.* 1958). Although it is possible to determine the relative humidity of the air within the drum it is not possible to set the temperature; this is dependent on the external temperature around the apparatus.

2.2.4.3 Survival estimations

Standard volume air samples (10.5 l min^{-1}) were withdrawn for 1 min from the drum using impingers (AGI 30) containing 10ml volumes of saline. Samples were removed at intervals of between 15 and 30 min over a time course of 120 min. Samples were serially diluted in saline and aliquots spread on the surface of four nutrient agar plates per dilution. The plates were incubated for 48h at 37°C in air prior to estimating the number of colony forming units (cfu). The measured viable

count was expressed as a percentage of the expected count since the latter decreases in proportion to the volume of air removed from the drum by sampling.

2.2.4.4 Data analysis

Repeat experiments were performed and the mean percentage survival figures were plotted on an exponential scale against time (Fig. 2.2). In order to determine the death rate (D), a linear regression was carried out of natural log of cfu *versus* time. The gradient of this line indicates the decline in viable cells with time. This is then corrected (by subtraction) for decline in cell numbers due to the sampling process to calculate D (the fraction of cells dying per unit time). The average survival time ($t_{1/2}$) was then estimated by use of the formula:-

$$t_{1/2} = \frac{0.693}{D}$$

2.3 Results

2.3.1 Comparison of the tolerance of a range of *Salmonella* strains

The ability of twenty five selected *Salmonella* strains to grow under a range of adverse environments conditions is shown in Table 2.3. Marked differences were found in the ability of different serovars to grow under the test conditions. Most strains demonstrated a tolerance to high concentrations of sugars, with 92% of the strains growing on plates containing 40% glucose, and 88% in the presence of 50% sucrose. The strains of *S. pullorum* were the most sensitive to high sugar concentrations. Conversely, most strains were sensitive to ethanol, and only 20% of the strains could grow when 5% ethanol was included in the growth medium, with 3 of the 5 tolerant strains being of the serotype *S. enteritidis*. Most strains (68%) were unable to grow when the concentration of sodium chloride in the medium was increased to 7.5%; all of the strains which were able to grow were of the serotype *S. enteritidis* except for one *S. hadar* strain. However, variation was evident in the sensitivity of *S. enteritidis* PT4 strains to salt with only 50% of the strains growing on a medium containing 7.5% NaCl; all other *S. enteritidis* phage types tolerated this concentration of sodium chloride.

A distinctive pattern of tolerance was obtained with extremes of pH and temperature; these factors were found to positively discriminate for strains of *S. enteritidis*. Only strains of *S. enteritidis* PT4 and PT13a were able to grow at pH 4 and pH 10. None of the test organisms grew well at 5°C; however, weak growth was obtained with nine of the eleven *S. enteritidis* strains. In contrast, all the representative strains of *S. enteritidis* grew at 48°C, although some only weakly; one strain of *S. hadar* was the only other isolate capable of growth at this temperature.

The eight representative strains of *S. enteritidis* PT4 behaved similarly in their tolerance patterns, although strains SE25 and NCTC 132344 showed sensitivity to more factors, including pH 10, high levels of NaCl and ethanol; strain SE25 was also sensitive to 5°C. The strains of *S. enteritidis* PT13a gave the same tolerance patterns as the phage type 4 group. In general, strains of *S. enteritidis* showed greater resistance to the adverse conditions, especially to pH, temperature and sodium chloride. In contrast, the most sensitive serotype was the avian-adapted *S. pullorum*.

2.3.2 Comparison of general growth properties of the *Salmonella* strains

The doubling times of the strains varied over the range 24-115 min in VBM medium. Strains of *S. typhimurium* were able to grow most rapidly; the slowest rates were obtained with strains of *S. pullorum* (MGT=115 min) and *S. gallinarum* (MGT=111 min). The *S. enteritidis* PT4 strains split into two groups with respect to their growth rates; one group had doubling times in the range 39-48 min whilst the other group grew more slowly and cells divided approximately every hour.

All the strains were able to grow under an atmosphere of 10% CO₂:10% H₂:80% N₂. The majority of strains (89%) were serum resistant; however, the *S. pullorum* strains were sensitive to serum killing. No marked differences were identified in the surface hydrophobicity of the strains. Only the aflagellate serotypes *S. pullorum* and *S. gallinarum* gave negative motility results. The only serotype which did not demonstrate resistance to lysozyme was *S. gallinarum*.

2.3.3 Survival in aerosols

During the tests, the mean relative humidity was $74.9 \pm 2.9 \%$, $n = 7$, and the temperature $23.9 \pm 1.3^{\circ}\text{C}$, $n = 7$. A comparison of the ability of plate-grown and broth-grown cells of *S. enteritidis* PT4 226 405 and plate-grown *S. typhimurium* Swindon to survive in aerosols under the conditions described is shown in Fig. 2.2. *S. typhimurium* showed negligible loss of viability over the 120 min sampling period, thereby showing a greater ability to survive in aerosols than *S. enteritidis* 226 405. *S. enteritidis* PT4 cells previously grown on nutrient agar plates were better able to survive in aerosols than broth-grown cultures. As no loss of viability was observed with *S. typhimurium* over the test period, an average survival time could not be calculated. Estimates of the average survival time for plate-grown and broth-grown cells of *S. enteritidis* PT4 cells were 1271 ± 1470 and 33 ± 17 min, respectively (two repeats each). There are a number of factors affecting aerosol survival, making this technique more appropriate for comparative studies than for the determination of absolute values.

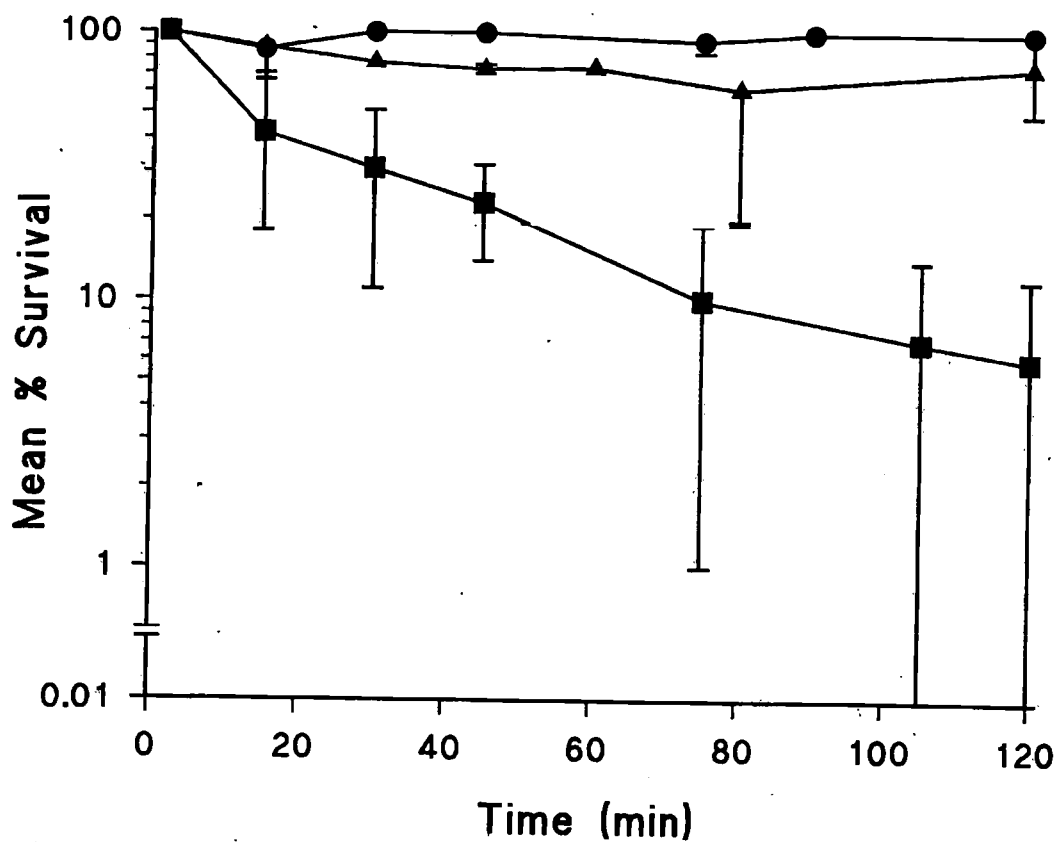
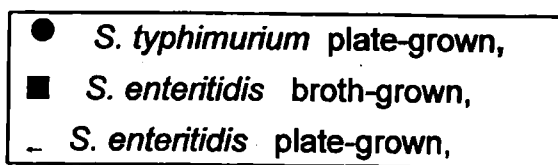


Figure 2.2 Aerosol survival of *S. enteritidis* PT4 and
S. typhimurium Swindon

Table 2.3 Detection of growth of salmonellae in hostile conditions.

ORGANISM *	ENVIRONMENTAL FACTOR							
	pH		TEMP. °C		NaCl	SUCROSE	GLUCOSE	EtOH
	4	10	5	48	7.5%	50%	40%	5%
<i>S. enteritidis</i> PT4								
NCTC P132344/1	+	+	w	w	+	+	+	-
NCTC P132344	+	-	w	w	-	+	+	-
NCTC P227166	w	+	w	+	+	+	+	-
NCTC P230666	+	+	w	+	+	+	+	-
C6B - hen isolate	w	+	w	w	+	+	+	+
226 405- human isolate	+	+	w	+	-	+	+	+
SE25 - Australian isolate	+	-	-	w	-	+	+	-
SE30 - Australian isolate	+	w	w	w	-	+	+	-
Other PTs of <i>S. enteritidis</i>								
NCTC P 265593 - PT 8	-	-	w	w	+	+	+	+
NCTC P102936 - PT 13a	+	+	-	+	+	+	+	-
NCTC P202267 - PT 13a	+	+	w	+	+	+	+	-
<i>S. typhimurium</i>								
STM 1116 - chicken isolate	-	-	-	-	-	+	+	-
STM Bangor - chicken isolate	-	-	-	-	-	+	+	-
STM Swindon - human isolate	-	-	-	-	-	+	+	-
<i>S. choleraesuis</i>								
Essex - pig isolate	-	-	-	-	-	+	+	-
B195 - pig isolate	-	-	-	-	-	+	+	-
A50 - pig isolate	-	-	-	-	-	+	+	-
<i>S. pullorum</i>								
NCTC 10704	-	-	-	-	-	-	-	-
NCTC 10706	-	-	-	-	-	-	+	-
<i>S. hadar</i>								
S 2656/81	-	-	-	w	-	+	+	-
1429/80	-	-	-	-	+	+	-	-
Other serotypes								
<i>S. gallinarum</i> NCTC 9240	-	-	w	-	-	+	+	-
<i>S. virchow</i> NCTC 5742	-	-	-	-	-	-	+	+
<i>S. infantis</i> chicken isolate	-	-	-	-	-	+	+	+
<i>S. kedougou</i>	-	-	-	-	-	+	+	-

+ = growth
 w = weak growth
 - = no growth
 EtOH=ethanol

* Strains provided by Dr. B. Rowe, CPL,
 Dr. T Humphrey, Exeter PHL. and Dr. P. Barrow,
 Institute For Animal Health, Compton.

Table 2.4 Inherent growth properties of a range of *Salmonella* strains.

ORGANISM	INHERENT PROPERTIES				
	DOUBLING TIME IN VBM (MIN)	MOTILITY	SAT* (M)	LYSOZYME RESISTANCE	SERUM RESISTANCE
<i>S. enteritidis</i> PT4					
NCTC P132344/1	41	+	2.0	R	R
NCTC P132344	44	+	2.0	R	R
NCTC P227166	65	+	2.0	R	R
NCTC P230666	39	+	2.0	R	R
C6B - hen isolate	48	+	2.0	R	S
226 405 - human isolate	45	+	2.0	R	R
SE25 - Australian isolate	59	+	2.0	B	R
SE30 - Australian isolate	60	+	2.0	R	R
Other PTs of <i>S. enteritidis</i>					
NCTC P 265593 - PT 8	55	+	2.0	B	R
NCTC P102936 - PT 13a	50	+	2.0	R	R
NCTC P202267 - PT 13a	37	+	2.0	R	R
<i>S. typhimurium</i>					
STM 1116 - chicken isolate	36	+	2.0	R	R
STM Bangor - chicken isolate	43	+	2.0	B	R
STM Swindon - human isolate	24	+	2.0	R	R
<i>S. choleraesuis</i>					
Essex - pig isolate	68	+	1.0	B	R
B195 - pig isolate	51	+	1.4	B	R
A50 - pig isolate	97	+	1.4	R	R
<i>S. pullorum</i>					
NCTC 10704	53	-	2.0	R	S
NCTC 10706	115	-	2.0	R	S
<i>S. hadar</i>					
S 2656/81	60	+	2.0	R	R
1429/80	50	+	2.0	R	R
Other serotypes					
<i>S. gallinarum</i> NCTC 9240	111	-	2.0	S	R
<i>S. virchow</i> NCTC 5742	52	+	1.0	R	S
<i>S. infantis</i> chicken isolate	49	+	2.0	R	R
<i>S. kedougou</i>	45	+	2.0	R	R

* SAT salt agglutination test -hydrophobic bacteria aggregate at lower salt concentrations

+ = motile

R = resistant

S = sensitive

B = bacteriostatic

2.4 Discussion

Since the mid 1980's, the incidence of foodborne disease associated with *S. enteritidis* has increased whilst the prevalence of other serotypes of *Salmonella* has remained relatively constant (Fig. 1.2). This has, in part, been associated with an increase in the frequency of carriage of *S. enteritidis* by poultry leading to contaminated poultry products entering the human food chain (Sections 1.5 and 2.2.1). Changes in eating habits and poor appreciation of the requirement for good hygiene practises have also been implicated as contributory factors in the observed increase in foodborne disease (Section 2.1.2). Since *S. enteritidis*, and specifically, certain phage types including PT4, have been so successful at persisting and out-competing other organisms, it may be anticipated that some properties of *S. enteritidis* will differ from those of other types of *Salmonella*, particularly in their resistance to environmental conditions and/or expression of virulence determinants.

Little variation was observed in the surface properties of the bacteria under evaluation. All serotypes were motile under the test conditions, except *S. pullorum* and *S. gallinarum* which have been considered to be aflagellate. A recent investigation of the poultry pathogen, *S. pullorum*, has indicated that motility can be induced and that long surface appendages similar to flagella are produced under certain conditions (Holt and Chaubal 1997).

The hydrophobic nature of cells has been associated with attachment to host tissues and with interactions between bacteria and phagocytes (Rosenberg *et al.* 1980). The ammonium sulphate aggregation test is a simple technique by which the hydrophobicity of bacterial cell surfaces can be inferred (Lindahl *et al.* 1981). Hydrophobic bacteria tend to aggregate at lower salt concentrations due to the interplay of electrostatic and van der Waals forces between cells. At higher salt concentrations, the influence of electrostatic stabilisation disappears and van der Waals interaction between bacteria causes aggregation. A study of a range of clinical *Salmonella* strains demonstrated that the majority of isolates had low cell surface hydrophobicity (Baloda and Krovacek 1991). Only serotypes *S. virchow* and *S. choleraesuis* Essex aggregated at salt concentrations below 1.4M in this

study (Lindahl *et al.* 1981). Hydrophobicity has been shown to be dependent on the growth conditions and phase of growth (Onaolapo and Klemperer 1986); therefore, the estimations made in this investigation only reflect the hydrophobicity of cells under the conditions prevailing during the test. However, little difference is observed between the test strains in terms of surface hydrophobicity.

Most strains tested showed resistance against lysozyme activity, indeed, only one strain of *S. gallinarum* was sensitive to lysozyme activity (Table 2.4). This may indicate that the significance of naturally-occurring lysozyme e.g. within eggs, as an inhibitor against contamination with salmonellae is limited.

A large proportion (84%) of the test strains were also resistant to the killing action of serum under the conditions used. Resistance to serum may be important in pathogen survival during disease in humans since, recently-isolated strains of *E. coli* from patients with diarrhoea and some strains of *S. typhimurium* demonstrated serum resistance (Pramoonjago *et al.* 1992). No change in the biology of *S. enteritidis* PT4 has been associated with the increased colonisation of chickens (Cox 1995), and little variation was observed in the surface associated properties of the *Salmonella* serotypes included in this study.

In contrast, distinct patterns for growth under extreme environmental conditions were observed among the different salmonellae. In general, strains of *S. enteritidis* were able to grow over a wider range of extreme conditions. Growth at extremes of pH and temperature positively discriminated for strains of *S. enteritidis*. Phage types 4 and 13a were the only groups to survive these pH challenges. The stress responses of *S. enteritidis* PT4 which determine these survival properties may be mechanisms which enhance persistence and growth. These responses will ultimately contribute to its prevalence in human disease, for example, pH tolerance may be important in pathogenicity and survival in food products with high or low pH values. The eight representative strains of *S. enteritidis* PT4 behaved similarly in their survival patterns, although some strains, (SE25, an Australian strain, and NCTC 132344) were more frequently inhibited by extreme conditions. *S. enteritidis* PT13a also had a similar survival

pattern to that of the phage type 4 group, whereas the phage type 8 strain was more sensitive to pH challenges.

Variations in the tolerance of *S. enteritidis* PT4 strains to pH and temperature have been reported by others (Humphrey *et al.* 1996). Some variation reported between laboratories may be associated with different experimental techniques (Radford and Board 1995). Some strains of *S. enteritidis* show increased tolerance to a range of environmental stresses whilst others are consistently more sensitive to environmental challenges. Strains of *S. enteritidis* PT4 which had increased tolerance to heat were shown to also survive better than sensitive strains when exposed to pH 2.6, 10mM hydrogen peroxide or to the drying effects when in droplet form on a surface (Humphrey *et al.* 1995).

At present, Australia does not have a significant problem with *S. enteritidis* infections in humans and few poultry flocks are infected with this serotype (Lightfoot, D. 1993). The Australian strains of *S. enteritidis*, reference SE30 and SE25, were amongst the more sensitive and slower-growing strains of *S. enteritidis* PT4. These results suggest that the reduced prevalence of *S. enteritidis* in Australian poultry flocks may, in part, be linked to contamination of their flocks with less resistant types of *S. enteritidis*; and that only the more resistant types are capable of persisting at all levels of the poultry industry and consequently of causing human disease. A larger number of strains would need to be compared, before conclusions concerning the properties of Australian strains could be drawn.

Associations between increased tolerance and increased virulence have been made. It was observed that virulent strains of *Salmonella* had a greater tolerance to acid challenge when compared to an avirulent laboratory strain (Lee, I.S. *et al.* 1995; Bearson *et al.* 1996). Isolates of *S. enteritidis* with an increased resistance to heat and pH were more virulent in mice and more invasive in chickens (Humphrey *et al.* 1996). The most sensitive serotype was the avian-adapted, *S. pullorum*, which has a high incidence in poultry, and low incidence in human disease (Barrow *et al.* 1994). As only a small number of representative strains of

the different serotypes were used, definitive conclusions cannot be made concerning each serotype.

Most infections occur on or through mucous surfaces (Smith 1995). To be pathogenic, the organisms must be able to grow at these *in vivo* interfaces. The main source of nutrients in the host are endogenous. An ability to grow more rapidly in minimal conditions where mucin is a source of energy and carbon would confer a selective advantage to an infecting organism. Strains of *S. typhimurium* had the most rapid growth rates with doubling times (DT) ranging from 24-43 minutes in Vogel Bonner medium supplemented with mucin (VBM), *S. enteritidis* PT4 strains had the next fastest growth rates, although strains could be split into two groups, one with doubling times in the range 39-48 minutes and the other with slightly slower doubling times (approximately every 60 minutes). A correlation of increased growth rate with increased tolerance was not observed within the small number of strains included in this investigation. However, a correlation of enhanced resistance with increased prevalence in human disease is indicated.

Initially, the effects of growth phase and growth conditions on the survival of *S. enteritidis* PT4 in aerosols were compared, since growth conditions and metabolic activity of bacteria are known to affect their ability to survive in the airborne state (Hambleton *et al.* 1983). For comparison of the serotypes, the more aerosol-resistant plate-grown cells were selected. Both *S. enteritidis* PT4 and *S. typhimurium* grown overnight on solid media were able to remain viable and aerosol-stable for longer, compared to mid-exponential broth-grown organisms. In natural environments, growth rates are usually sub-maximal (Tempest *et al.* 1984) and are often associated with a surface (Costerton *et al.* 1987). In this respect, plate-grown cells may provide a more relevant simulation than batch cultures. When plate-grown cells of *S. enteritidis* PT4 and *S. typhimurium* were compared directly for their aerosol stability, *S. typhimurium* remained viable in greater numbers than *S. enteritidis*. Indeed, *S. typhimurium* showed no detectable loss of viability at 75% RH over 2 h compared to a loss of approximately 30% viability for *S. enteritidis* over the same time period. Cells grown on solid media would be expected to have a lower mean metabolic activity

than their corresponding broth grown cells and this may have enabled them to survive longer. This has been demonstrated with other bacteria such as *Legionella pneumophila* (Costerton *et al.* 1987) and *Escherichia coli* (Benbough *et al.* 1972; Dark and Callow 1973).

The survival times in aerosols for plate-grown *S. enteritidis* PT4 and *S. typhimurium* compared favourably with other organisms. Under similar conditions of temperature and relative humidity, aerosols of *L. pneumophila*, an organism pathogenic via the airborne route, were shown to have only 10% viability after 2h and preliminary data on *Mycobacterium tuberculosis* showed only 7% viability after 30 min (M. S. Lever, unpublished data).

The ability to survive in aerosols and other stressed environments may contribute to the pathogenic potential of the cells. Studies performed on *L. pneumophila* suggested that more virulent isolates were better able to survive in aerosols (Hambleton *et al.* 1983). Recent work has also shown that two strains of *S. enteritidis* PT4, with varying virulence in mice and invasiveness in chickens, showed differences in their ability to survive in aerosols (Humphrey, *et al.* 1996), with the more virulent isolate being able to survive for longer. This airborne stability, therefore may be selecting for more virulent strains of *Salmonella* within environments such as henhouses.

The potential for salmonellae to be disseminated via the airborne route in significant numbers in certain practices and situations has been reported. Humphrey *et al.* have demonstrated the ability of *S. enteritidis* to survive in thin dry films of either batter or egg on Formica work surfaces within the splatter zone in domestic kitchens when eggs are whisked (Humphrey *et al.* 1994). Gustavsson and Borch have also shown that dehiding and chilling in cold storage rooms were implicated as critical processing steps with respect to aerosol contamination of beef carcasses with Enterobacteriaceae (Gustavsson and Borch 1993). A recent study has demonstrated that airborne infection of hens with *S. enteritidis* can occur between hens 1m apart, and that this route of transmission may be associated with induced molting (Holt *et al.* 1998).

Faecal pellets from populations of mice in hen houses have been shown to contain high levels of *S. enteritidis* (2.3×10^5 cfu per total pellet) (Henzler and Opitz 1992) and provide a reservoir of infection. This number of organisms in pellets exceeds the infective dose reported by Baskerville *et al.* 1992, who showed that hens became infected and developed systemic infection with an airborne challenge of 4.2×10^3 bacteria (Baskerville *et al.* 1992). A similar infective dose (10^3 bacteria) by conjunctival challenge also resulted in systemic infection demonstrating the potential role for airborne infection in henhouses (Humphrey *et al.* 1992).

In conclusion, this study has shown that both *S. enteritidis* PT4 and *S. typhimurium* are capable of surviving in aerosols, for long periods of time, and in numbers greater than required to produce systemic disease in chickens, implying that the airborne route may be relevant for the transmission of *Salmonella*.

pH and temperature were the most discriminatory environmental factors to select for *S. enteritidis*; and pH was particularly discriminatory for PT4 and PT13a strains. As these phage types are amongst the most frequently isolated from human infection world-wide, identifying and understanding the contribution of pH resistance to their prevalence may provide general insights into the pathogenesis of *S. enteritidis*. Consequently the effect of pH on the physiology, survival and virulence of *S. enteritidis* PT4 was investigated further and the findings are described in the following chapters.

Chapter 3

The Effect of Environmental pH on the Physiology and Surface Structures of *Salmonella enteritidis* PT 4.

3.1 Introduction

The data described in Chapter 2, show *S. enteritidis* strains have an enhanced ability to grow under hostile conditions, when compared to other serotypes of *Salmonella*. In addition, the more prevalent phage types (4 and 13a) were generally able to grow at pH values which were inhibitory to the other serotypes tested (Table 2.3). The pH values in natural environments frequently differ from optimum for growth and survival of *S. enteritidis* (near neutrality). Consequently, the greater tolerance of *S. enteritidis* PT4 to shifts in pH will increase the survival potential of the organisms and, therefore, the chances of initiating infection.

A remarkable characteristic of bacterial cells is their ability to grow under a wide range of conditions, and it has long been recognised that these conditions influence the physiology of bacteria (Schaechter *et al.* 1958). Consequently, the milieu from which *S. enteritidis* is derived will influence the physiology of cells and possibly the expression of surface structures.

3.1.1 Flagella

Most natural strains of *S. enteritidis* PT4 are motile, moving rapidly in liquid medium and being capable of abrupt directional changes. The flagella which are responsible for the movement can be viewed by electron microscopy and are long, thin surface appendages randomly spaced around the cell; the number of flagella per bacterium varies. Environmental conditions have been shown to modulate motility in a range of bacterial species e.g. *Bordetella bronchiseptica* (Akerley *et al.* 1992) and *Campylobacter coli* (Alm *et al.* 1993). The role of flagella and motility in the virulence of *S. enteritidis* is still to be determined.

The flagellar system has two components: the rigid surface structure and a

biochemical network which relays chemical and physical information concerning the environment to the flagellar 'motor', which controls the rotation of the flagella. The functioning of the two components in synchrony enable bacteria to move in response to diverse environmental stimuli (Macnab 1996).

Synthesis of bacterial flagella and the accompanying array of chemotaxis receptors and transducers represents a major commitment of energy and resources by growing bacteria (Helmann 1991). Spontaneous aflagellate mutants of *S. typhimurium* have been shown to overtake motile cells, in stirred cultures due to the metabolic cost of synthesising the flagellar apparatus (Macnab 1996). Flagellar biosynthesis requires the regulated expression of nearly 50 gene products in a cascade which is linked to discrete morphological signals (Macnab 1996).

3.1.2 Fimbriae

It has been hypothesised and in some cases demonstrated that fimbriae are important for the pathogenicity of Gram-negative bacteria in general and members of Enterobacteriaceae in particular (Finlay and Falkow 1989). Passage of *Salmonella* species from the mucosal surface to the underlying tissues involves attachment to the epithelium of the gastrointestinal tract followed by invasion of intestinal epithelial cells (Giannella *et al.* 1973; Foster and Spector 1995). Enteric fever, gastro-enteritis and bacteraemia all require an invasion stage. Attachment to epithelial cells, colonisation via site-specific receptors, and evasion of host defences are functions which have been associated with bacterial surface structures. Evidence also suggests a role in the survival of *S. enteritidis* by enveloping the bacteria in hydrophobic fimbriae which are resistant to changes in temperature, pH and water activity.

The classification of *Salmonella* fimbriae has been based upon their morphology and their ability to mediate erythrocyte agglutination in the presence or absence of α D-mannose (Duguid *et al.* 1966). *S. enteritidis* cells express an array of fimbrial antigens composed of protein polymeric units. The numerical nomenclature of the fimbrial type refers to the size of the major subunit, e.g. 14KDa, 17KDa, etc.

- SEF 21 fimbriae are members of the type 1 class; they were first described on

strains of *S. enteritidis* in 1991, (Müller, K.H. *et al.* 1991). It is postulated that these fimbriae are expressed when bacteria are in close association with cells rather than in an extracellular environment. However, there is no conclusive evidence that type 1 fimbriae of *S. enteritidis* possess an adhesin or are involved in colonisation of epithelial surfaces (Thorns 1995).

- SEF 14 fimbriae were identified relatively recently and are unique to certain serotypes of *Salmonella* including *S. enteritidis*, (Thorns 1995). Their role remains unclear although it has been suggested that they do not contribute to the pathogenesis of *S. enteritidis* in mice or chickens (Thorns *et al.* 1996; Ogunniyi *et al.* 1997).
- SEF17 fimbriae were first described on *S. enteritidis* by Collinson *et al.*, (Collinson *et al.* 1991), they have recently been associated with a “lacy” colony morphology in *S. enteritidis* and may be associated with increased virulence (Guard-Petter *et al.* 1996; Humphrey *et al.* 1996).
- Other less well characterised fimbriae of *S. enteritidis* include SEF18, long polar fimbriae (LPF), plasmid encoded fimbriae (PEF) and bundle-forming pili (BFP) (Woodward *et al.* 1996).

To date most studies have concentrated on SEF 21 fimbriae; the roles for each fimbrial type and their interrelationships are yet to be determined. The expression of fimbriae has been shown to be regulated by environmental and cell signals; however, the mechanisms behind this are unclear.

3.1.3 Plasmid carriage

A role for plasmids in the virulence of salmonellae was first identified in 1982 (Jones, G.W. *et al.* 1982). Several studies have identified the presence of a 38MDa plasmid in many strains of *S. enteritidis*, which was serotype specific (Helmuth *et al.* 1985), and which has become associated with virulence in mice (Nakamura *et al.* 1985; Hovi *et al.* 1988; Chart *et al.* 1989; Suzuki *et al.* 1992). See also **Section 5.1.3.4.**

3.1.4 Continuous culture

Prolonging the growth of bacteria by the continuous addition of fresh medium has been discussed for more than half a century. As early as 1946, Gerhardt

designed an apparatus in which the rate of addition of fresh medium and the withdrawal of product was continuous and simultaneous during the culture of *Brucella suis* (Gerhardt 1946).

The basic principles of continuous culture are that fresh medium is added at a constant rate to a perfectly mixed culture of bacteria, the volume of which is maintained constant by the culture being harvested at the same rate. The growth rate of the culture is determined by the availability of a limiting nutrient which is controlled by the rate of addition of the medium. The basic theory of continuous culture identified the possibility of fixing the specific growth rate at any value between zero and the maximum by using a pre-determined rate of medium addition (Monod 1950; Novick and Szilard 1950). The application of continuous culture is essential in order to distinguish between the effects of growth rate and changes in environmental parameters. In simple batch systems, the cultures are always in a transient state as nutrients become depleted and end-products accumulate. Essentially, the chemostat method simplifies the culture system to facilitate the elucidation of the reaction of organisms to their environment (Pirt 1975).

Consequently, continuous culture techniques were adopted for this investigation as they provide numerous advantages over conventional batch cultures. Specifically:

- reproducible samples of culture can be obtained over large periods of time,
- the growth rate can be controlled and maintained constant,
- individual environmental parameters can be varied and their influence can be determined
- cultures are not subject to the considerable changes in conditions that occur in batch cultures,
- large volumes of steady-state cells can be obtained by collecting the effluent from the culture vessel.

3.1.5 Steady-state

If a chemostat system is left undisturbed for sufficient time a “steady-state” culture

is obtained; this is where 'balanced growth' occurs i.e. the exponential growth rate and the proportions of enzymes, nucleic acids and other essential metabolic and structural components of the cell are constant (Pirt 1975). Steady-state theory is based on the concept that in a constant environment the rate of cellular functions is constant if the growth rate is maintained constant. This self regulation causes the proportion of cellular components to vary in response to environmental shifts to maintain the constant growth rate.

Ten pot volume changes of medium (or ~five days, at $D=0.1 \text{ h}^{-1}$), is the time required before a culture is considered to have reached steady-state. This time can be confirmed as being sufficient for the organisms to stabilise to the culture conditions by mathematical considerations. When the medium is flowing at a rate which gives a dilution rate of 0.1 h^{-1} , the mean generation time is 6.9h (see below); the time required for ten pot volume changes of medium is 100h. To ensure that this value was exceeded, the cultures were left undisturbed for 5 days (120h). Therefore, the number of cell divisions in this period was

$$= \frac{120}{6.9} = 17.4.$$

At each division any components accumulated from the previous growth environment will be halved. Therefore, after 17 divisions the level of the component will be reduced by a factor $= 0.5^{17} = 7.6 \times 10^{-6}$.

If the original concentration is considered to be 100%, then after 5 days the remaining concentration would be 0.0007% of the original, which can be considered as negligible. If time is required after inoculation for the culture to achieve the required volume before dilution takes place, the 5 day period is sufficiently in excess to cover this.

3.1.6 Growth rate

Salmonellosis requires the multiplication of cells within a host and the rate at which the bacteria can divide *in vivo* is fundamental to the host-pathogen interaction. Estimations of the *in vivo* growth rate are difficult to determine. It is likely that the growth rate would not remain constant during infection but would fluctuate as more and less limiting conditions are experienced. The consensus of investigations into *in vivo* growth rate is that the bacteria divide at sub-maximal

rates. The division rate of *S. typhimurium* in the spleen of artificially infected mice was estimated to be in the range of once every 5-10 h (Maw and Meynell 1968). When this is compared to the 30 min doubling time obtained in laboratory culture it can be seen that the organisms *in vivo* are dividing at a rate which is only 5-10 % of their maximum.

3.1.7 Oxygen availability

In many areas of the host there are exceptionally low levels of oxygen tension giving almost anaerobic conditions. Only small quantities of air are swallowed into the gastrointestinal tract, and any oxygen from this source or from the cells lining the tract is metabolised by the commensal microflora. Consequently, the lower regions of the gastrointestinal tract have negative oxidation-reduction potentials; the redox potential in the human colon is about -200mV (Tannock 1995). Therefore, cultures were grown under anaerobic conditions during this investigation.

3.1.8 Mucin

Mucus is constantly secreted by goblet cells, forming a layer along the gastrointestinal tract. It is a complex mixture containing mucin glycoprotein which is an important source of carbon and energy for commensal mucin-degrading bacteria. The mucus layer forms an efficient, physical barrier against invasion and colonisation by bacteria by inhibiting attachment to the mucosal cells (Beachey 1981). The degradation of the mucins reduces the protective effect and also liberates nutrients. There is a dynamic balance between mucin secretion and utilisation, the outcome of which has an important effect during infection. Growth within the mucus layer was necessary for successful colonisation of the colon of mice by *E. coli* and *S. typhimurium* (Wadolowski *et al.* 1988).

The mucin used in this study was from the porcine stomach and its principal components are hexosamine (37%), hexoses (27%), fucose (10%), protein (20%) and neuraminic (sialic) acid (6%) (Glenister *et al.* 1988).

3.1.9 Aims

The aim of this study was to determine the role of environmental pH as a signal for regulating the expression of potential virulence factors of *S. enteritidis* PT4. Surface structures including fimbriae and flagella of bacteria are the first point of interaction between a pathogen and the host, and have been postulated to influence attachment to epithelial cells (**Sections 3.1.1 and 3.1.2**). The majority of strains of *S. enteritidis* PT4 isolated from cases of human enteritis have been shown to carry the 38MDa plasmid (Threlfall *et al.* 1994) and this plasmid has been associated with the virulence of *S. enteritidis* in mice (**Section 3.1.3**). Consequently, the approach of this investigation was to use a continuous culture system with conditions defined to be similar to those encountered within a host e.g. sub-maximal growth rate, mucin-supplemented medium, and low oxygen and nutrient availability, to determine the range of pH over which a clinical isolate of *S. enteritidis* PT4 could grow. Then, using growth at the limits of the pH range and neutrality for comparisons, to determine the effect of growth pH on:

1. growth (yield and maximum growth rate) of *S. enteritidis* PT4,
2. the expression of surface structures (fimbriae and flagella),
3. the carriage of the 38 MDa plasmid.

3.2 Methods

3.2.1 Bacterial strain

A clinical isolate of *S. enteritidis* PT4, reference 226 405, was selected for investigation. This strain was isolated from the faeces of a patient during a small outbreak of foodborne salmonellosis by Salisbury Public Health Laboratory. Strain 226 405 was able to grow at extreme pH values and also belonged to the group of *S. enteritidis* PT4 strains which could grow more rapidly, (**Tables 2.3 and 2.4**).

3.2.2 Growth conditions

To determine the pH range for growth and the effect of environmental pH on bacterial cell properties, strain 226 405 was grown in a chemostat (LH 500 series II, LH Engineering, Inceltech, Reading, Berkshire) with a 500ml working volume,

in VBM medium, (made as described in **Tables 2.1 and 2.2**), under an atmosphere of 5% CO₂ in nitrogen (B.O.C London), at 37°C. To minimise damage to surface structures, whilst still providing adequate mixing, the culture was stirred at 500 rpm using a magnetic stirring bar. The medium volume was kept constant by an overflow weir. The medium flow rate was set at $50 \pm 2 \text{ ml.h}^{-1}$ to give a dilution rate of 0.1 h^{-1} .

$$\text{The dilution rate (D) = } \frac{\text{the medium flow rate}}{\text{culture volume}} \quad \text{h}^{-1}$$

$$\text{Mean generation time (MGT) = } \frac{\log_{10} 2}{D} = \frac{0.693}{D} \quad \text{h}$$

Therefore, a dilution rate of 0.1 h^{-1} is equivalent to a mean generation time of 6.9 h. The pH in the culture vessel was maintained at the required value by the automatic addition of 2M NaOH or 2M HCl. The pH of the culture was continuously monitored by incorporating an autoclavable, combination pH electrode (Ingold, Mettler Toledo, Bowmontleys) into the chemostat vessel. The pH range for growth was determined by increasing or decreasing the pH level until steady-state cultures could no longer be maintained.

Steady-state conditions were allowed to establish in the chemostats, and the purity of the cultures was confirmed daily by spreading an aliquot of the culture onto nutrient agar and XLD plates. Viable counts were also estimated on a daily basis to confirm that the viable cell number stabilised when the culture was assumed to be in steady-state and was in agreement with previous chemostat cultures grown under identical conditions. Through-out the chemostat run medium flow rate, pH and temperature were monitored to ensure all conditions remained stable.

3.2.3 Growth parameters

At each pH level studied, the culture was allowed to achieve steady-state conditions before use (five days at this dilution rate, (**Section 3.1.5**)). Purity was confirmed daily by spreading an aliquot of the culture onto nutrient agar plates which were incubated at 37°C in air for 2 days. Any variant colonies were

confirmed as *S. enteritidis* on selective media: XLD (bioMerieux UK Ltd., Basingstoke) and DCA Hynes (Lab M, Bury, Lancs.) *Salmonella* colonies are black on these media. Cell yields were determined daily by estimation of viable counts on nutrient agar after overnight incubation at 37°C. Dry weight and optical density measurements were not used as measures of cell yield as the mucin content of the medium interfered with these parameters.

The maximum specific growth rate (μ_{\max}) was determined by the wash-out method (Tempest 1970b). The medium flow rate into the chemostat was increased until the bacteria were flowing out of the culture at a rate faster than they could be maintained by cell division. When the dilution rate (D) is greater than the maximum growth rate of the bacteria (i.e. $D > \mu_{\max}$), the number of bacteria within the culture starts to decline. The rate of decline of cell number is proportional to D minus μ_{\max} (i.e. how much faster the dilution rate is than the maximum growth rate). After the flow rate had been increased, the decline in cell number was followed by viable counts on nutrient agar plates over a 20 min time period. A value for μ_{\max} was estimated by plotting $\log N$ against time, where N is the viable cell number; the gradient of the line is equal to (μ_{\max} minus D). Since D is proportional to the flow rate of the medium, μ_{\max} can be calculated. Several estimations of μ_{\max} were made using different dilution rates and the maximum observed value was selected as being the best estimate of μ_{\max} for these environmental conditions (3.2.2). μ_{\max} estimations were performed on cultures soon after steady-state was achieved to minimise errors which might occur if wall growth present in the culture vessel sloughed off during the estimation period. Measurements of the early decline in cell numbers after increasing the flow rate means that the culture viable count is still high and any contribution to viable cell numbers made by wall growth is obviated.

3.2.4 Motility estimations

Motility was assessed by light microscopy using a Thoma counting chamber (Hawksley, London). The number of motile bacteria within a counting square was estimated using fresh culture samples from each steady-state. 1% formaldehyde w/v was then added to kill cells prior to a total count being made within the same counting field. Motility was expressed as a proportion of the total cell count.

Motility proportions estimated from chemostat cultures were compared with cells grown in static Vogel Bonner medium (pH 7), at 37°C for 18 hours, in batch cultures.

3.2.5 Surface structures

Cell morphology and surface structures were viewed using transmission electron microscopy. Culture samples were fixed overnight by the addition of 0.5% w/v formaldehyde, applied to formvar/carbon filmed, 400-mesh EM specimen grids using a disposable plastic loop which avoided cell damage from shear forces. Grids were negatively stained using 1% w/v phosphotungstic acid prior to visualising the cells.

3.2.6 Environmental transitions

The proportion of motile cells and the distribution of surface structures were also estimated after switching a culture from one pH value to another. Initially, the chemostat culture was allowed to reach steady-state at pH 9.45; pH control was then discontinued and, after 24h, the pH stabilised at pH 7.10 and a new steady-state was allowed to establish. Cells were taken directly from the chemostat before, during and after this transition, and were assessed for motility and surface structures as described above.

3.2.7 Plasmid content

After 10 days growth at each steady-state, the plasmid profiles of cultures taken directly from the chemostat were determined and carriage of the 38-MDa plasmid was assessed. A modification of the method described by Portnoy *et al.* was used (Portnoy *et al.* 1981), with extra care taken in the preparation of the alkaline denaturing solution (Casse *et al.* 1979), while the plasmid was precipitated from the neutralised, cleared lysate using polyethylene glycol (Humphreys *et al.* 1975). Plasmid DNA was analysed by agarose-gel electrophoresis using 0.7% (w/v) agarose gels containing 0.05 µg ml⁻¹ ethidium bromide and performed in Tris-acetate buffer (Tully 1991). Gels were then examined using U.V. transillumination. Plasmids in *Escherichia coli* 39R861 were also included as plasmid size reference molecules (Threlfall *et al.* 1986). *S. enteritidis* PT4 strain

p132344 and an isogenic variant *S. enteritidis* p132344/1, which is plasmid free, were also included as controls (Chart *et al.* 1989).

3.2.8 Data analysis

Data on cell yields between chemostat runs at the same pH value and between different pH values were analysed by two-way analysis of variance, using Statgraphics (STSC Version 5.0, Inc., Rockville, MD, USA). Pairwise comparisons between cell yields at the three pH values were made using the Scheffé test. Differences were assumed to be significant at $p < 0.05$.

The proportions of cells with surface structures at the three different pH levels were compared using the large samples proportions test (assuming a normal distribution) (Fisher and Van Belle 1993). Differences were again assumed to be significant at $p < 0.05$.

3.3 Results

3.3.1 Growth characteristics of *S. enteritidis* PT4 strain 226 405

Steady-state growth was possible in the chemostat over the range pH 4.35-9.45 under the conditions described; steady-state growth was not possible outside of this pH range. Without pH control, the culture stabilised at pH 7.10. Therefore, this pH and the two extremes of pH (4.35 and 9.45) were selected for comparative studies. The reproducibility of repeat chemostat runs was good, with no significant difference being detected between three independent runs using two-way analysis of variance ($F_{[10,82]} = 1.59$, $p = 0.125$). Cell yields at the different pH levels were compared by two-way analysis of variance and were highly significantly different ($F_{[2,10]} = 404.1$, $p < 0.0001$). Following the analysis of variance, pair-wise comparisons of each combination of pH levels were made using the Scheffé test. Each pair-wise comparison of cell yields was significantly different ($p < 0.001$), with most of the variation due to greatly reduced cell yields being obtained at the extremes of pH compared to pH 7.10 (Table 3.1).

The observed maximum growth rate was reduced at pH 4.35; such a reduction was not observed at pH 9.45, where the doubling time was 35 mins, the same as that measured at pH 7.10 (Table 3.1).

Table 3.1. Characteristics of *S. enteritidis* PT4 strain 226 405 grown in continuous culture at different pH values

Growth pH	Cell Numbers (log ₁₀ cfu.ml ⁻¹)	Maximum Growth Rate (μ _{max}) h ⁻¹	Maximum Doubling Time (min)
4.35	7.24 ± 0.19 [n=27]	0.99	42
7.10	8.63 ± 0.15 [n=45]	1.20	35
9.45	6.96 ± 0.45 [n=22]	1.20	35

μ_{max} = maximum growth rate.

Cell numbers are expressed as mean viable counts ± standard deviation values.

n = the sample size

3.3.2 Cell motility

A reduction in the proportion of motile cells was observed during continuous culture; approximately 90% of batch grown cells were motile, compared with only approximately 10% in continuous culture at pH 7.10. The proportion of motile cells was reduced still further to approximately 1% during growth at the limits of pH. The percentage of motile cells correlated with the proportion of cells expressing flagella as determined by electron microscopy (Table 3.2).

Table 3.2 Motility and presence of flagella on cells of *S. enteritidis* PT4 grown under different culture conditions

Culture Type	pH	Percentage of bacterial cells that were	
		motile	flagellate*
Batch [n= 4]	7.10	>90	81± 9
Continuous [n=3]	4.35	1	2 ± 1
Continuous [n=2]	7.10	10	8 ± 4
Continuous [n=2]	9.45	<1	2 ± 2

* samples are means ± standard deviation values
n = sample size

3.3.3 Surface structures

Growth at the extremes of pH in the chemostat had marked effects on the distribution of cell surface structures estimated by EM (Table 3.3). For each culture condition repeat samples were visualised and at least 120 cells examined, generally, 200-250 cells were counted. The proportion of cells with SEF 21 was considerably reduced at pH 9.45. Figure 3.3 shows a typical cell from the culture growing at pH 9.45; at this pH value, 95% of the population had no surface structures, whereas only 42% of cells were devoid of structures when grown at a constant pH 7.10. Figure 3.2 illustrates a cell growing at pH 7.10 expressing fimbriae, but no flagella. The structure of the fimbriae (size and straightness) are typical of type 1 (SEF 21) fimbriae. A reduction in surface structures was also observed at pH 4.35, but to a lesser extent; 69% of this population were 'bald'. Figure 3.1 is a transmission electron micrograph of a thin section of an *S. enteritidis* PT4 cell grown at pH 4.35 and illustrating an exaggerated convoluted appearance of the cell membrane. Figure 3.4 illustrates a batch grown cell of

The proportions of cells expressing fimbriae were shown to be significantly different at the three pH values when the data were analysed using the large sample proportions test, with $p < 0.001$ for all comparisons; most of the difference was between the extremes of pH and pH 7.10. A comparison of pH 7.10 against pH 4.35 and pH 9.45 gave $Z = 9.17$, $p < 0.00001$ and $Z = 23.4$, $p < 0.00001$, respectively. The proportion of cells expressing fimbriae was also shown to be significantly different between pH 4.35 and pH 9.45, $Z = 4.30$, $p < 0.00002$. A higher variation in the estimation of surface structures was observed with the low pH culture. Mucin was observed to be less soluble at low pH values and this may result in the surface of the cells being coated, leading to less efficient staining and visualisation of structures. The proportion of flagellate cells was significantly higher at pH 7.10 than at pH 9.45, ($Z = 4.96$, $p < 0.00001$); however, there was no significant difference when compared to pH 4.35 ($Z = 0.55$, $p < 0.60$). The proportion of cells with flagella was marginally higher at pH 4.35 than at pH 9.45; $Z = 2.18$, $p < 0.03$.

3.3.4 The effect of a transition in pH on the expression of flagella

The surface structures of cells were monitored during a transition from steady-state growth at pH 9.45 to pH 7.10. Standard chemostat theory no longer applies during transition periods between steady-states (Monod 1950). Chemostat theory would only apply if all cell functions responded in a uniform way in response to the disturbing factor. It is more likely that the various mechanisms of cell metabolism and growth respond at differing rates to the changing environment (Young *et al.* 1970). At pH 9.45, prior to the transition, no cells were observed expressing flagella when viewed by electron microscopy and $<1\%$ of the population were estimated to be motile by light microscopy. Within 1.5 h of discontinuing pH control, the pH had fallen to pH 8.79 and the viable count at this point had increased to 6.0×10^6 cfu.ml⁻¹. After a further 1.5 h, the pH had reached pH 8.4, the viable count had increased to 1.5×10^7 cfu.ml⁻¹ and the percentage of the population expressing flagella (by EM) was now 18%. No further estimations were made until a steady-state was achieved at pH 7.10, when the majority of cells expressed fimbriae (Table 3.3).

Table 3.3 Surface structures detected on cells of *S. enteritidis* PT4 grown in continuous culture at different pH values

growth pH	Mean percentage of bacterial cells with			
	fimbriae only	flagella only	both fimbriae and flagella	neither
Steady state				
4.35	20 ± 15 [n=2]	11 ± 12 [n=2]	0 [n=2]	69 ± 41 [n=2]
7.10	52 ± 16 [n=4]	6 ± 4 [n=4]	1 ± 1 [n=4]	42 ± 14 [n=4]
9.45	3 ± 2 [n=4]	2 ± 2 [n=4]	0 [n=4]	95 ± 1 [n=4]
Transition				
9.45 (0)*	5	0	0	95
8.4 (3.0 h)*	6	18	12	76
8.2 (4.5 h)*	17	20	0	75

n = the number of culture samples viewed for each condition. A minimum of 120 cells were examined by EM in each sample; generally between 200-250 cells were counted.

* surface characteristics of cells grown initially at pH 9.45 after pH control was discontinued the number in brackets is time in hours after pH control ended (See section 3.3.4).

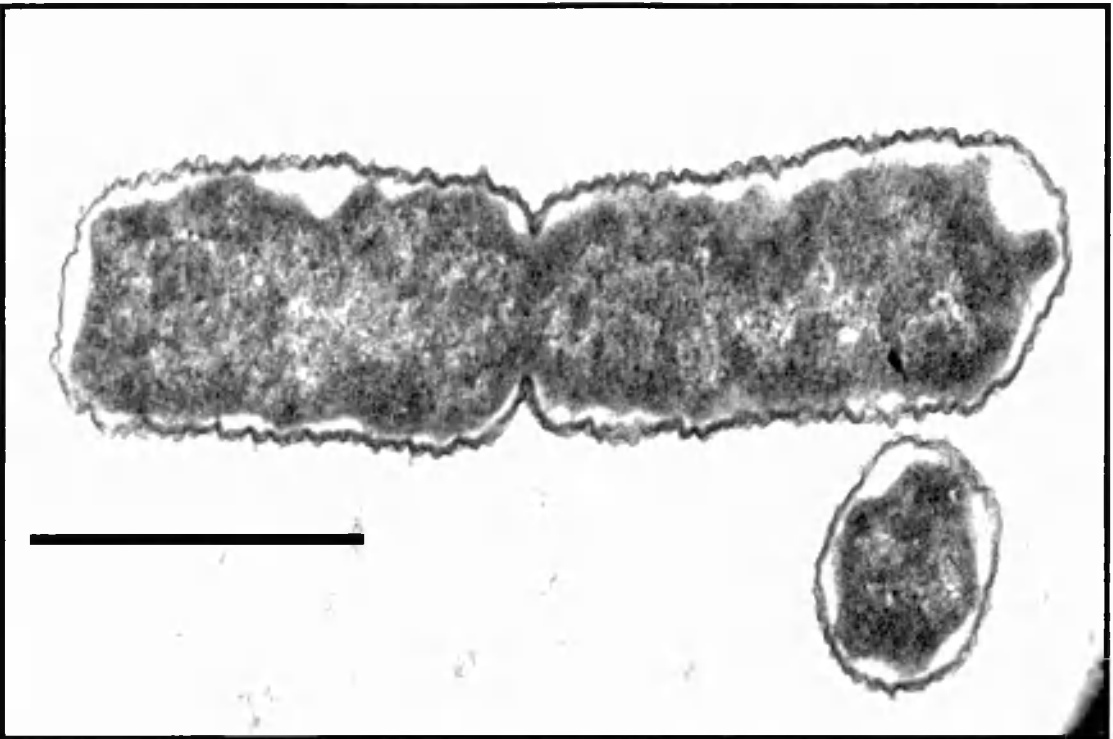


Figure 3.1 Transmission electron micrograph of a thin section of *S. enteritidis* PT4 grown continuously at pH 4.35 showing bacterial cells with convoluted wall morphology.

Bar=1 μ m

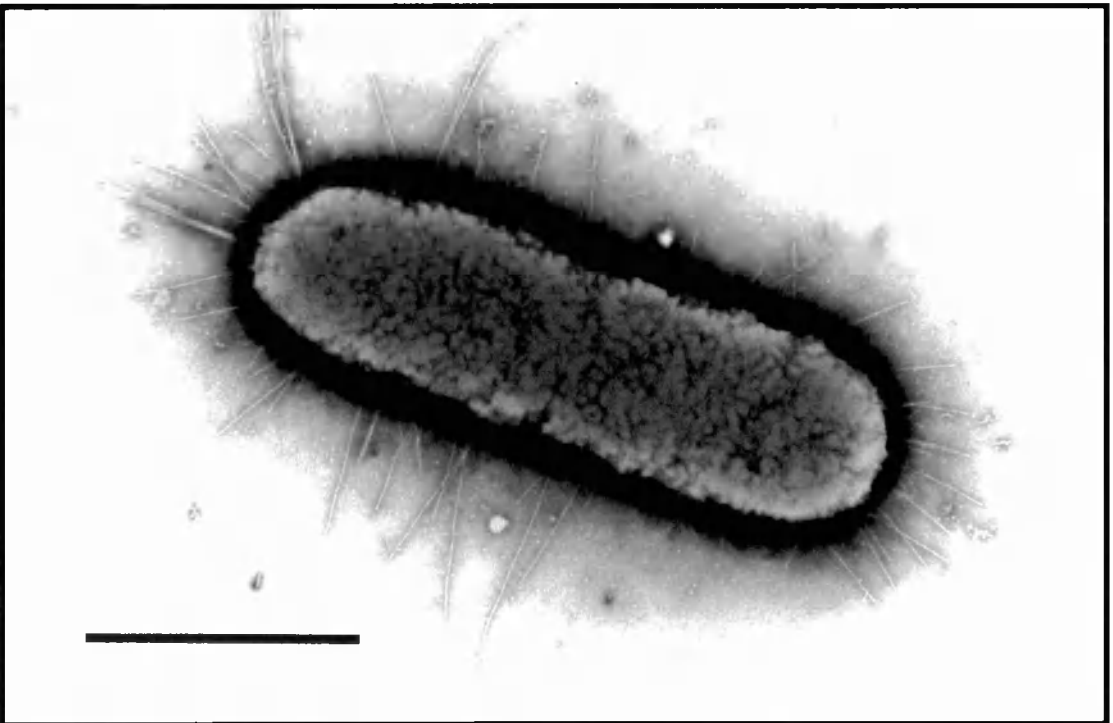


Figure 3.2 Transmission electron micrograph of *S. enteritidis* PT4 grown in continuous culture at pH 7.1 showing cells with many fimbriae

Bar = 1 μ m

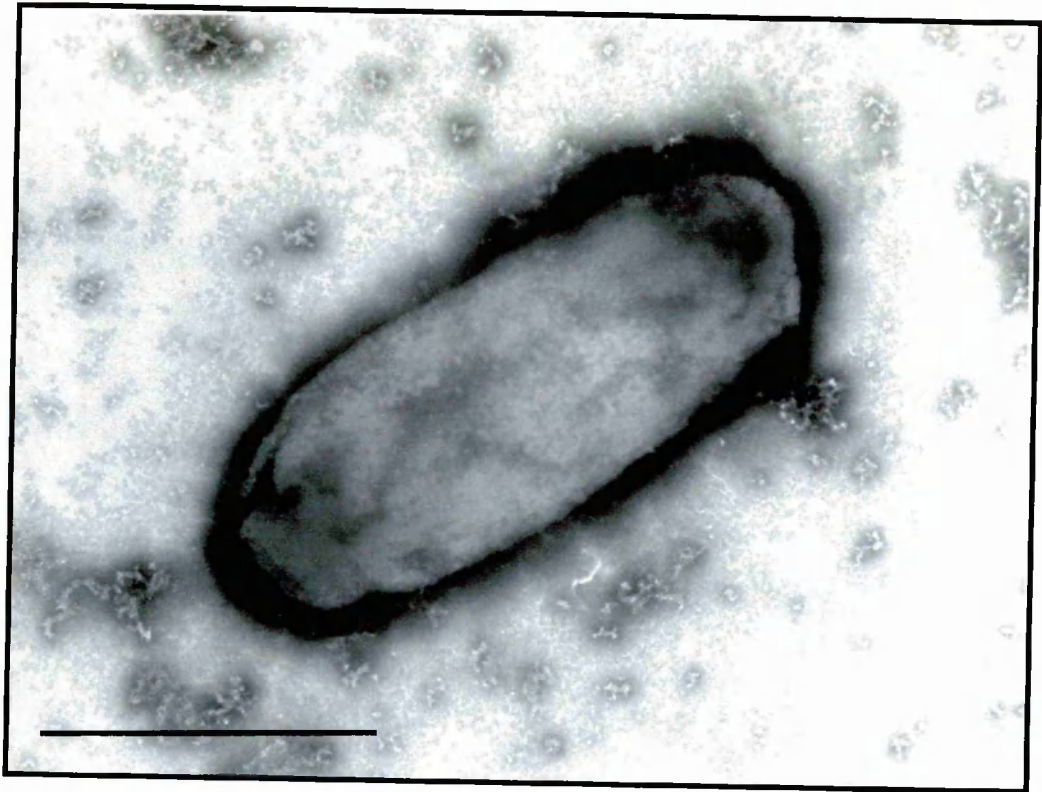


Figure 3.3 Transmission electron micrograph of *S. enteritidis* PT4 grown at pH 9.45 showing a bacterial cell with no surface structures. Bar=1 μ m

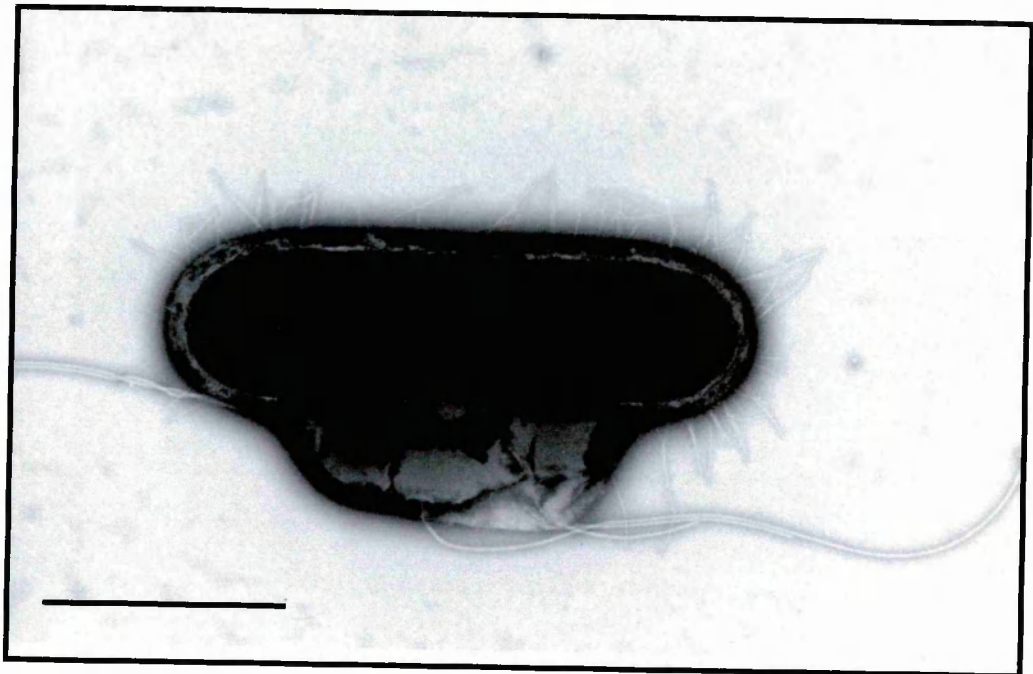


Figure 3.4 Transmission electron micrograph of *S. enteritidis* PT4 grown in batch culture at pH7 expressing both fimbriae and flagella. Bar=1 μ m

Electron micrographs kindly prepared by A B Dowsett

3.3.5 Plasmid content

The 38 MDa plasmid was present in cells that had been grown continuously for 10 days in steady-state and in overnight batch cultures at pH 7.10. However, this plasmid was not detected in cultures growing continuously at either pH 4.35 or pH 9.45 (**Fig. 3.5**). The plasmid profiles of fresh and stored cells (-20°C), for each pH, were compared, and no differences were observed.

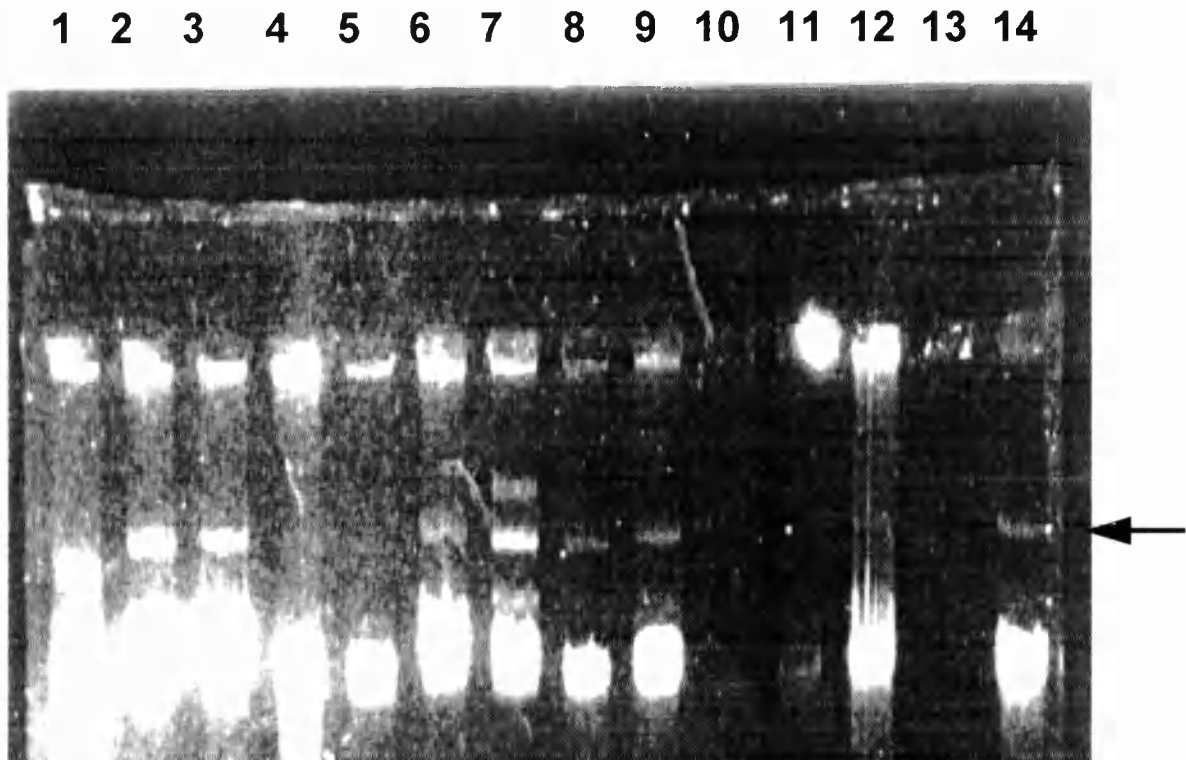


Figure 3.5 Detection of plasmid DNA by agarose electrophoresis

S. enteritidis PT4 strain 226 405 was grown to steady-state in continuous culture at pH 4.35, 7.10 and 9.45 (these are the chemostat samples referred to below); stored samples were kept at -20 °C prior to use:

Lane 1, MW ladder; Lane 2, *P. aeruginosa* strain RP4 - containing 36 MDa plasmid; Lane 3, *S. enteritidis* p132344; Lane 4, pH 9.45 chemostat - stored sample; Lane 5, *S. enteritidis* p132344/1; Lane 6, pH 7 batch culture, L-broth; Lane 7, *E. coli* 39R861; Lane 8, pH 7.10 chemostat - stored sample; Lane 9, pH 7.10 chemostat - stored sample; Lane 10, pH 9.45 chemostat; Lane 11, pH 4.35 chemostat; Lane 12, pH 9.45 chemostat; Lane 13, pH 4.35 chemostat; Lane 14, pH 7.10 chemostat.

3.4 Discussion

A clinical isolate of *S. enteritidis* PT4, strain 226 405 was able to sustain growth over the pH range pH 4.35 to pH 9.45. This range is of relevance as it corresponds with the extremes of pH predicted to be encountered by this organism in the environment, in food products, and during infection. For example, the pH of ovalbumin reaches pH 9.5 during storage of intact eggs (Board *et al.* 1994), whereas mayonnaise, which has been repeatedly implicated as a vehicle for *S. enteritidis* infection, can have a pH of around 4.0 (Perales and Garcia 1990; Lock and Board 1995).

Most bacteria of importance in foodborne diseases are unable to grow below about pH 4.5. This means that acidic foods do not normally present major health risks as far as the common bacterial agents causing food-transmitted disease are concerned (Mossel *et al.* 1995). The minimum pH for growth of *E. coli* and *S. paratyphi* was estimated to be pH 4.4 and pH 4.5, similar to those determined in this study. However, the maximum pH allowing growth of these two strains was pH 9.0 and pH 7.8, respectively; therefore, the pH range allowing growth of these two strains is narrower than that determined for *S. enteritidis* PT4 in this study. Comparable data on the pH range for growth of other foodborne pathogens showed *Bacillus cereus* and *Staphylococcus aureus* to have similar ranges to those detected in this study, i.e. pH 4.9 - 9.3, and pH 4.0 - 9.8, respectively. Wider ranges of pH tolerance were observed for alkali-tolerant bacteria such as *Vibrio parahaemolyticus* where the limiting values for growth were between pH 4.8 and pH 11.0 (Commission on Microbiological Specifications for Foods 1980).

The strain used in the present study was isolated from the faeces of an infected patient. The ability of strains of *S. enteritidis* PT4 to grow over such a wide pH range may not be a universal property as cells of other isolates have been shown to lose viability at pH values below pH 5.0 (Chart *et al.* 1994). However, the results reported in **Table 2.3**, would indicate that a high proportion of isolates are able to grow over a wide range of pH values. Parameters compared between batch and continuous cultures may differ markedly, and many environmental factors e.g. medium type, available oxygen, will also influence the ability of cells to

survive pH stress (Radford and Board 1995). Prior exposure to environmental stress may alter the subsequent ability of cells to grow and survive. For example, a short incubation of *S. enteritidis* PT4 in media at pH values between 3 and 6 resulted in a marked and rapid increase in the acid resistance of some cells (Humphrey *et al.* 1993a). In contrast, cells with sub-lethal injuries induced by environmental stress factors may be more sensitive to pH extremes.

Plasma membranes are not freely permeable to hydrogen or hydroxyl ions. Consequently, intracellular and extracellular hydrogen ion concentrations do not necessarily equilibrate, and a concentration gradient across the membrane can be expected. Although the plasma membrane may provide some protection against external variations in pH levels it is well recognised that bacterial growth and metabolism is readily influenced by external pH (Booth 1985; Hall *et al.* 1995). The maximum growth rate at pH 4.35 was significantly reduced, and the mean generation time increased to 42 min; in contrast, a similar decrease was not observed at pH 9.45, where the doubling time was 35 mins. This observation may be explained, in part, by a greater requirement for energy to maintain intracellular pH near to neutrality when the culture is growing at pH 4.35. The value of μ_{\max} determined in VBM at pH 7.10 under anaerobic conditions was faster than the growth rate determined in batch culture under air (**Table 2.4**), i.e. 35 and 45 min, respectively.

Growth at extremes of pH had significant effects on surface structures and plasmid carriage of cells. The proportion of motile cells during continuous culture was only a fraction of those in batch cultures. This may be due to shear forces, the absence of nutrient gradients with continuous mixing, or cell energetics, as organisms with the lowest energy requirements tend to be selected in continuous cultures (Pirt 1975). Synthesis of bacterial flagella and the accompanying array of chemotaxis receptors represents a major commitment of energy and resources in growing cells. The proportion of motile cells in cultures at the extremes of pH was very low (~1%), and this value closely correlated with the number of flagellate cells. This suggests that the loss of motility was due to a shut down in the synthesis and assembly of flagella. Similar observations have been made in

Campylobacter jejuni in which the expression of the sigma 54 flaB flagellin promoter was subject to environmental regulation. FlaB filament protein production was affected by growth pH, the composition of the growth medium, and the temperature of growth (Alm *et al.* 1993).

The formation of flagella has been shown to depend on the growth phase of the bacterium and suggestions of a regulatory connection between flagellar synthesis and cell division genes has been made (Harshey and Toguchi 1996). The low production level of flagella in continuous culture in comparison to batch cultures may therefore be linked to the different growth kinetics of the two systems. Repression of the production of flagella has also been demonstrated in *S. enteritidis* PT4 in response to low pH conditions (Chart *et al.* 1994).

Environmental pH had significant effects on the distribution of fimbriae on cells in our study. Only 3% of cells growing at pH 9.45 had fimbriae compared to 20% at pH 4.35 and 52% at pH 7.10. These findings are in agreement with observations made on the production of fimbrial adhesins by enterotoxigenic *E. coli* during growth at various specific growth rates and at different pH values (van-Verseveld *et al.* 1985). A decrease in the production of fimbriae at pH values both above and below pH 7 was also seen; the production of fimbriae was also dependent on the growth rate of cells, with a significant production of fimbriae only detected at specific growth rate values higher than 0.2 h^{-1} , although no significant differences were observed between aerobic and anaerobic growth (van-Verseveld *et al.* 1985).

Salmonellae express many different types of fimbrial antigens; *S. enteritidis* has been shown to possess structures differentiated as SEF 14, SEF 17, SEF 18, SEF 21 (type 1) and plasmid encoded fimbriae (PEF) (**Section 3.1.2**). There is also evidence of bundle-forming pilus and long polar fimbriae, although the total number of structures is still to be confirmed (Collinson *et al.* 1993; Thorns *et al.* 1996). To date, research has tended to focus on the role of each fimbrial type in isolation, in particular type 1 (SEF 21). However, the role of fimbriae in the pathogenesis of *S. enteritidis* remains unclear, and the picture may be confused

by different types of fimbriae acting in concert or performing similar functions. Finlay *et al* demonstrated that the induction of several *Salmonella* proteins was required for *S. typhimurium* and *S. choleraesuis* to adhere to and invade epithelial cells (Finlay *et al.* 1989).

In the present study, it was not possible to determine the effect of pH on the individual fimbrial types, and only SEF 21 was considered; it is possible that particular fimbriae respond differently to specific environmental stimuli. The expression of SEF 14 has been shown to be influenced by the type of growth medium, and in one strain, SEF 14 fimbriae were produced in peptone water at pH 7.2 but not at pH 6.0 (Woodward *et al.* 1993).

The 38MDa plasmid in *S. enteritidis* has been associated with virulence in mice (Chart *et al.* 1989), but the relevance of the virulence plasmid to human infection remains unclear. The plasmid was detected in this strain when cells were grown continuously at pH 7.10; however, it was not detected in cells grown continuously at pH 4.35 or at pH 9.45. The use of plasmid profiling for epidemiological investigation necessitates stable plasmids. Most studies of plasmid stability have been concerned with *in vivo* stability of plasmid profiles for the duration of outbreaks. The 38MDa plasmid is reported to be highly stable (Olsen *et al.* 1994a), but it may be that extreme environmental conditions of pH and nutrient availability induce changes in plasmid content and expression (Caulcott *et al.* 1987) and reduce the proportion of plasmid-carrying cells in the population to below the detection level of the assay. Without direct selection, plasmid-containing bacteria may survive as only a small minority of the population.

In order to determine whether the absence of motility and flagella during growth at pH 9.45 was a phenotypic response rather than a genotypic effect, the presence of flagella on cells and motility was monitored when pH control was discontinued and the pH fell from 9.45 to 7.10. Flagella began to reappear on cells almost immediately suggesting that this was indeed a phenotypic reaction, and that the expression of the relevant genes can be rapidly switched on or off in response to environmental stimuli.

In conclusion, *S. enteritidis* PT4 could grow over a wide range of pH values, including those that are found in foods associated with outbreaks of disease. pH was found to markedly regulate the expression of surface structures and the carriage of a 38MDa plasmid, and both of these factors have been implicated in the virulence of *S. enteritidis*. Bacterial pathogenesis requires the expression of various virulence factors at different stages of infection; this would imply that the control of each factor at the individual level would be optimal. Consequently, the pathogenic potential of cells grown at different pH values will be compared in a mouse model, and the data described in **Chapter 5**. To cause infection, *S. enteritidis* must be able to survive and replicate in the distinct anatomical compartments of the host; therefore, it is also important to consider the effect of environmental growth pH on the stress tolerance of *S. enteritidis* PT4, and this will be addressed in the following chapter (**Chapter 4**).

Chapter 4

An Evaluation of the Interdependency of Selected Stress Responses in *S. enteritidis* PT4

4.1 Introduction

Ocean and field pollution due to the introduction of raw sewage has increased steadily, producing tremendous changes in natural habitats. Bacteria are under considerable pressure to adapt and habituate to environmental stresses in order to survive such changes and compete against other organisms. A complex interaction of global climate changes, increased ultraviolet radiation and pollution with human and animal waste, in various environmental sites, may hasten the rate of mutation and genetic transfer in enteric microbes, potentially enhancing their ability to survive.

Environmental factors can have significant effects upon the gene expression of bacteria (Patrick and Larkin 1995), thus modulating their persistence and virulence. Expression of surface structures and carriage of the 38MDa plasmid by *S. enteritidis* PT4 was influenced by growth pH (Chapter 3) illustrating that the physiology of *S. enteritidis* PT4 is pH-responsive. Presumably, these responses work towards enabling the bacteria to maintain their intracellular pH at a level where cellular functions can continue, and viability can be maintained.

4.1.1 Factors affecting bacterial tolerance

The ability of bacteria to survive extreme conditions is dependent on a range of factors:-

1. growth phase (Foster and Spector 1995; Humphrey *et al.* 1995)
2. growth conditions (Peters *et al.* 1991; Lin *et al.* 1995)
3. previous stress exposure (Humphrey 1990b; Humphrey *et al.* 1991a, 1993b; Leyer and Johnson 1992, 1993; Rowbury *et al.* 1993a, b; Foster 1995)

4. other environmental conditions during the stress challenge (Peters *et al.* 1991; Radford and Board 1995; Rowbury 1995).

4.1.2 Adaptation

The ability to sense and respond to environmental factors is of crucial importance in bacterial survival. Cells of *S. enteritidis* must be able to adapt to a broad spectrum of stresses encountered in the natural environment (Section 1.11), in the food chain (Table 1.1) and also during infection when a battery of antimicrobial agents are encountered (Section 5.1.1). The nature and levels of environmental stresses may be subject to rapid fluctuations and are frequently experienced either sequentially or in combinations. To adapt to a dynamic environment or to passage through a series of environments bacteria must be able to modulate gene expression at the transcription level. Organisms must possess either constitutive or adaptive protection strategies. Generally, genes contributing to environmental adaptation are not expressed constitutively but are activated in response to an environmental signal.

The effect of applying two or more inhibitory factors is usually either additive or synergistic. However, when bacteria elicit a stress response to the initial stimulus some degree of cross-protection against other environmental extremes, and/or enhanced tolerance to subsequent exposures to the stimulating factor, may be conferred. The consequences of bacterial adaptation have major implications in the food industry and during infection; for example pre-adapted *S. enteritidis* cells may persist better on food products and within a host.

4.1.3 Cross-protection between stress factors

The defence mechanisms used to survive environmental stresses can be specific or global (Foster and Spector 1995). The genes controlling the stress response possess a highly specific regulator, and possibly also a more pleiotrophic regulator (Dorman 1994), which enables regulation of a series of overlapping genes to occur simultaneously.

Genes which are regulated by multiple systems may respond to various environmental cues and, consequently, may have a role in the survival strategy of

bacteria exposed to multiple extreme factors. For example, the production of 'heat shock' proteins is also promoted by starvation, oxidative shock and acid treatment (Patrick and Larkin 1995). The existence of pleiotrophic control mechanisms which affect the expression of very large sub-sets of genes providing global regulation of environmentally-regulated responses, explains the interdependency of some stress responses.

4.1.4 Acid adaptation

Acidic pH is one of the stresses most frequently encountered by microbes. An ability to adapt to low pH conditions will, in part, determine the persistence of *S. enteritidis* in both free living and pathogenic situations. To survive acid challenge, bacteria must be able to maintain their intracellular pH using phenotypic responses. An adaptive response to acidic conditions has been demonstrated in many Gram-negative bacterial species (Nojoudi *et al.* 1995).

Detailed investigations of the adaptive responses of *S. typhimurium* to low pH have identified a number of distinct systems (Lee, I.S. *et al.* 1994). Exposure to moderate acid stress induced the synthesis of proteins that increased survival during subsequent more severe acid challenge; the survival process has become known as the acid tolerance response, (ATR) (Foster and Hall 1990). These responses include inducible systems for acid growth and log-phase and stationary-phase acid tolerance responses, a summary of which is shown in **Table 4.1**.

The alternate sigma factor, sigma S (σ^S) which is the product of the *rpoS* locus, is associated with late-log-phase and is required to direct the transcription of stationary phase stress survival genes (Foster 1995), and has been shown to play a role in acid survival (Small *et al.* 1994; Lee, I.S. *et al.* 1995), (**Table 4.1**). Mutants defective in *rpoS* display only modest, transient levels of tolerance (Lee, I.S. *et al.* 1995).

Table 4.1 A summary of the acid tolerance responses of *S. typhimurium*

Growth phase	pH level for induction	Role for RpoS	Cross-protection	References
log	pH 5.8	dependent	includes: crystal violet, heat & salt	(Leyer and Johnson 1993; Foster 1995; Foster and Spector 1995; Bearson <i>et al.</i> 1996)
log	≤ pH 4.5	dependent-sustained response. independent-transient response	includes: heat, H ₂ O ₂ , osmolarity	(Foster and Spector 1995; Lee, I.S. <i>et al.</i> 1995)
stationary	pH independent	dependent	general stress resistance response	(Lange and Henngge-Aronis 1991; Hengge-Aronis 1993)
stationary	low pH (maximum induction at pH 4.3)	independent		(Lee, I.S. <i>et al.</i> 1994)

Habituation to low pH has also been demonstrated in *S. enteritidis* PT4 following short-term exposure to mild acid levels. A marked increase in acid tolerance was induced following several minutes exposure to pH levels in the range pH 3.0-6.0 (Humphrey *et al.* 1993a). Habituation involved two separate systems one of which was independent of protein synthesis (Humphrey *et al.* 1993a).

4.1.5 Alkali-adaptation

Enterobacteria are also commonly exposed to alkaline conditions in polluted waters, in sewage and in some foods e.g. egg whites. Also, during infection, mildly alkaline conditions may persist at times in phagolysosomes and the intestine (Sections 1.11 and 5.1.1). Adaptation to high pH conditions has been demonstrated in *E. coli* (Goodson and Rowbury 1990; Rowbury *et al.* 1996). *E. coli* cells grown at pH 9 had an enhanced ability to survive subsequent high pH challenges, which were lethal to non-adapted cells (Goodson and Rowbury 1990). Alkali-habituated cells had a greater resistance to DNA damage when exposed to pH 11 than cells grown at pH 7 and generally had enhanced repair mechanisms (Rowbury *et al.* 1989).

4.1.6 Selection of environmental stress factors

The cross-protection provided by pH adaptation against other inhibitory factors was assessed using *S. enteritidis* PT4. Challenges with lethal levels of pH, temperature and osmolarity (using NaCl concentrations) were selected as these are amongst the stresses most frequently experienced by *S. enteritidis* in food and during infection.

In order to survive high external osmotic pressure, the cell must be able to maintain its turgor; consequently, the surface structure of the cell in part determines the ability of organisms to survive osmotic stress. Crystal violet was included as a challenge factor as it is also active on the surface of bacteria. The lethal mode of action of crystal violet is due to the formation of an unionised complex of the bacteria with the dye (Adams 1967). The surface of bacteria is the first point of contact with external factors and plays a major role in the

environmental responsiveness of the cell. Therefore, the response to crystal violet and osmotic stress will provide additional information concerning the role of the cell surface in the adaptation of *S. enteritidis* to pH.

4.1.7 Aims

The effect of pH adaptation on the subsequent tolerance of *S. enteritidis* PT4 to pH and temperature challenges has been investigated (Humphrey *et al.* 1991a). Previous studies have used short-term exposures to adapt the cells. However, when considering bacteria within the environment, food products or during infection, exposure to hostile conditions may continue for hours or even days. Limited replication also frequently occurs under these adverse conditions. Consequently, the aims of this study were to investigate the effect of the adaptation responses of *S. enteritidis* PT4 to growth at the limits of the pH range on the ability of cells to survive subsequent exposure to lethal levels of:

1. pH
2. temperature
3. osmolarity
4. crystal violet

The level of cross-protection to these factors conferred by pH adaptation will be assessed by comparing the tolerance of adapted and non-adapted cells. A comparison of cells adapted to low pH will also be made with those adapted to high pH.

4.2 Methods

4.2.1 Bacterial strain and growth conditions

A clinical isolate of *S. enteritidis* PT4, strain 226 405, was grown in continuous culture in Vogel Bonner minimal medium (Vogel and Bonner 1956) supplemented with vitamins and mucin (prepared as described in **Tables 2.1 & 2.2**), in an atmosphere of 5% CO₂ and 95% N₂ at 37°C. The medium flow rate was set to give a dilution rate of 0.1h⁻¹ which is equivalent to a mean generation time of 6.9h. Cultures were grown at pH 7.10 and at the limits of the pH range for growth i.e. pH 9.45 and pH 4.35. The pH of the culture was maintained at the

required value by the automatic addition of 2M NaOH or 2M HCl. These conditions were described more fully in **Section 3.2.2**.

4.2.2 Thermal tolerance

10ml samples of VBM were equilibrated to the required temperature (50°C or 60°C in a water bath, or 5°C in a cold room) prior to inoculation with either 0.1ml of pH 7.10 culture or 0.5ml of pH 4.35 or pH 9.45 culture, removed directly from the relevant chemostat. The greater dilution of the pH 7.10 chemostat sample was necessary to compensate for the higher cell number in this culture (**Table 3.1**) and to eliminate any additional protection due to an increased cell density (Druilhet and Sobek 1976; Sitnikov *et al.* 1995). The initial cell density was constant for each challenge at approximately 10^6 cfu.ml⁻¹. The pH of all cultures was returned to neutrality by dilution. This technique avoids any time delay which would occur if the pH of the original sample was adjusted. To investigate the combined effect of pH and thermal stress, small samples were also removed directly from the chemostat into a water bath at the required temperature or a cold room; these culture samples remained at the growth pH. Small volumes were used in order to minimise the time required for the culture to reach the test temperature. Viable counts were followed with time. Samples of the culture were serially diluted in Maximum Recovery Medium and aliquots spread onto blood agar plates. Colony counts were made after 48h incubation at 37°C in air and viable cell numbers determined.

4.2.3 Tolerance to osmotic shock

A 2.5M solution of NaCl (Sigma, Poole, UK) was prepared in distilled water. 10ml samples were equilibrated at room temperature prior to inoculation with 0.1ml of culture grown at pH 7.10, or 0.5ml of chemostat cultures grown at either limit of pH. Cell viability was followed with time by performing viable counts as described above.

4.2.4 Tolerance to crystal violet

Fresh 10ml solutions of 0.0025% (v/v) crystal violet were prepared using saline and equilibrated to room temperature. The solutions were inoculated as described previously with 0.1ml of pH 7.10 culture or 0.5ml of pH 4.35 or pH 9.45

culture taken directly from the chemostat. The crystal violet solutions were maintained at room temperature throughout the challenge. Again, the number of survivors was monitored with time using viable counts as described above.

4.2.5 pH challenge

10 ml samples of Vogel Bonner medium were adjusted to various pH levels between pH 2.0 - pH 11, as shown in Table 2.1, by the addition of appropriate volumes of 2N HCl or 2M NaOH. These samples were inoculated as described previously with 0.1ml of pH 7.10 culture or 0.5ml of pH 4.35 or pH 9.45 culture taken directly from the chemostat. The samples were incubated at 37°C during the challenge period and the number of survivors was again determined by viable counts and was followed with time.

4.2.6 Data analysis

Survival curves were drawn for each stress factor by plotting \log_{10} % survivors against time. Decimal reduction times (D_{90} -values) were used to quantify the tolerance of the bacteria; this is defined as the time required to reduce the population number by one log cycle. D-values were calculated from the gradients of the survival curves. All tolerance experiments were repeated on at least three occasions and data from replicate experiments were used to calculate mean and standard deviation values. Pairwise comparisons of the tolerance of bacteria grown at different pH levels were made using the Scheffé test (Statgraphics, STSC version 5, Inc., Rockville, MD, USA). Differences were assumed to be significant at $p < 0.05$.

The death rate is described by the formula:

$$\frac{N}{N_0} = 1 - (1 - \exp(-kt))^n$$

where N_0 = initial cell number

N = number of survivors

t = exposure time

k = inactivation constant (or slope)

Average survival times were also estimated from plots of the natural logarithm of viable cell number against time. Initially, the death rate (decline in viable cell numbers with time) was determined by the equation:

$$N_t = N_0 e^{rt}$$

$$\ln N_t = rt \ln N_0$$

where r is equal to the average death rate.

The average survival time could then be determined using the formula:

$$\frac{\ln 2}{\text{death rate}}$$

4.3 Results

Some of the results are shown in different formats; survival curves are illustrated in **Figs 4.1-4.3**, and the D_{90} -values (decimal reduction values) calculated from the exponential region of the survival curves have been tabulated in **Tables 4.2-4.5**; average survival times which give an estimate of how long a bacterium survives the stress challenge are shown in **Table 4.6**. Some survival curves are included for visual comparison of resistance of the cultures grown at different pH levels. The rate of cell death was not always constant throughout the entire challenge period, but tended towards a more sigmoid relationship; the equation to describe sigmoidal destruction is complex (Mossel *et al.* 1995). Consequently, in this study the death rate was calculated from the linear section of the survival curves.

4.3.1 Thermal tolerance

All cultures demonstrated considerable resistance to low temperature; in fact, no decline in cell viability was observed during the test period in the cells which had been grown at pH 7.10. A decline in cell number was observed in the cultures grown at the extremes of pH, although the rate of loss of viability was low. The rate of decline in cell numbers at 4°C was not significantly different between pH 4.35 and pH 9.45 cultures (**Table 4.2**). Cells grown at the limits of the pH range were more resistant to 50°C than those grown at pH 7.10 ($p < 0.05$), (**Fig. 4.1** and **Table 4.2**). The thermo-resistance of the cultures grown at the limits of the pH

range were not significantly different at the 5% level, since the survival percentages of the two cultures fell within the variation of the technique. In all cases, cells survived temperature stress better when the pH was at neutrality (Table 4.2).

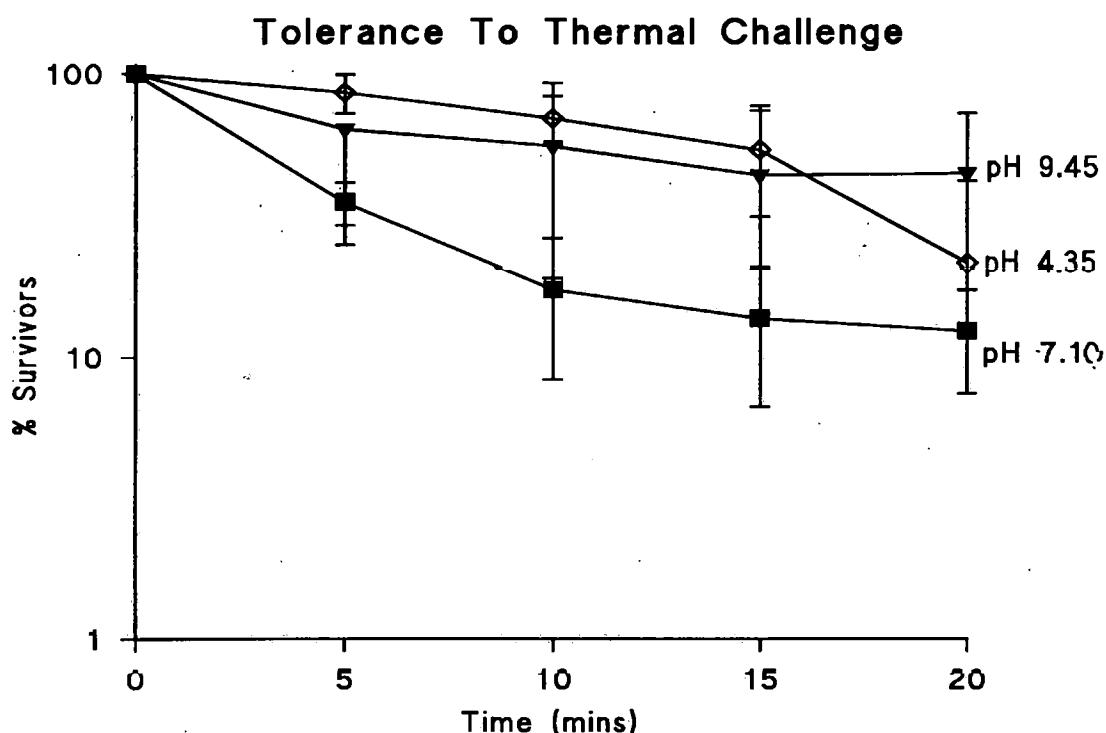


Figure 4.1 Survival curves for *S. enteritidis* PT4 when exposed to 50°C after growth at three pH values

4.3.2. pH tolerance

The three pH cultures survived exposure to a wide range of pH values, and no decline in viability was observed over the 20 min test period at pH levels between 3.5 and 9.5 (Table 4.3). Generally, acid-grown cells survived significantly longer during acid challenges. Cells grown at the extremes of pH had an enhanced ability to survive alkaline pH levels when compared to cultures grown at pH 7.10 (Table 4.3). No significant differences were observed in the pair-wise comparisons of D-values following pH 2 and pH 11 challenges. Cells grown at pH 9.45 survived for the longest when challenged at pH 10 or pH 11.

Table 4.2 The tolerance of *S. enteritidis* PT4 grown at three different pH values to thermal challenge

Growth pH	pH during the challenge	Mean killing time, D, at each challenge temperature (min)		
		4°C	50 °C	60 °C
4.35	4.35	140 ± 79 [n=6]	28 ± 9 [n=3]	0.9 ± 0.3 [n=4]
	7.00	324 ± 153 [n=3]	91 ± 17 [n=3]	1.8 ± 1.5 [n=6]
7.10	7.10	G	25 ± 6 [n=3]	ND
	7.00	G	23 ± 7 [n=3]	ND
9.45	9.45	144 ± 42 [n=4]	29 ± 9 [n=3]	0.7 ± 0.2 [n=2]
	7.00	258 ± 164 [n=4]	85 ± 13 [n=3]	1.2 ± 0.13 [n=3]

D = time to kill 90% of the population

ND = not determined

G = growth i.e. no decline in cell number

values are means ± standard deviation

* pairwise comparisons which were significantly different at $p < 0.05$ using Scheffé analysis. For clarity, only the comparisons which are significantly different are shown.

n=sample size

Table 4.3 Resistance of *S. enteritidis* PT4 grown at three different pH values to subsequent pH challenge

Challenge pH	Mean killing time, D* (min)		
	Growth pH		
	4.35	7.10	9.45
2.0			
	1.9 ± 0.9	1.0 ± 0.4	0.8 ± 0.4
	[n=4]	[n=5]	[n=4]
2.5			
	18 ± 9	3.3 ± 1.5	2.4 ± 2.0
	[n=4]	[n=4]	[n=4]
3.0			
	221 ± 143	21.2 ± 8.5	31.1 ± 13.8
	[n=4]	[n=5]	[n=6]
3.5-9.5	>120.0	>120.0	>120.0
10.0			
	37.6 ± 12.2	30.1 ± 4.7	50.8 ± 15.7
	[n=4]	[n=4]	[n=4]
11.0			
	2.5 ± 1.1	2.3 ± 1.3	3.5 ± 2.0
	[n=6]	[n=3]	[n=4]

D* = time to kill 90% of the population
values are means ± standard deviation

*shows pair-wise comparisons which are significantly different at the 5% level using Scheffé analysis.

4.3.3 Osmotic pressure (NaCl) tolerance

The resistance to high NaCl concentrations is dependent upon the bacteria surviving high osmotic stress and also low water activity. The level of resistance of the cultures grown at the three pH levels was markedly different (Table 4.4, Fig. 4.2). Cells grown at alkaline pH were significantly ($p < 0.05$) more sensitive to high levels of NaCl (Table 4.4, Fig. 4.2). Initially, the acid-grown cells showed enhanced resistance to NaCl activity although the rate of loss of viability subsequently became greater than that of cells grown at neutral pH (Fig. 4.2). The D-value for acid-grown cells was greater than that for pH 7 cultures, although not significantly so (Table 4.4).

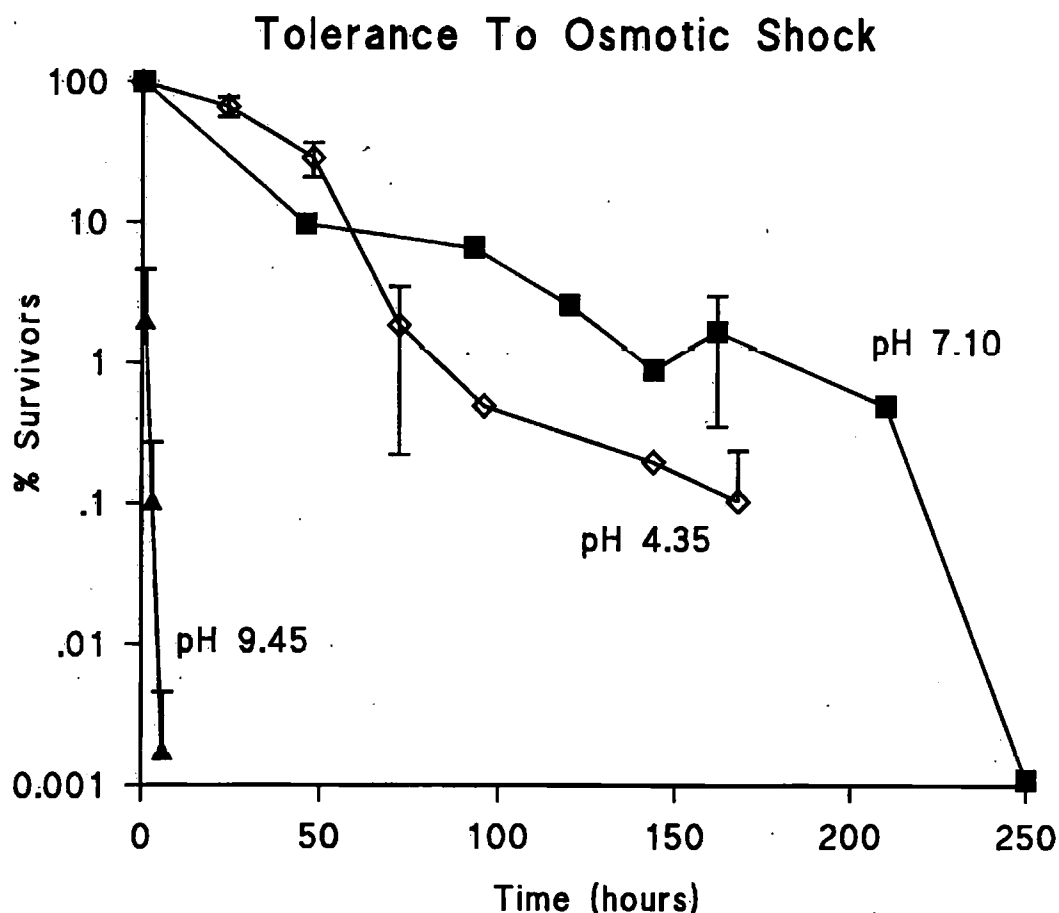


Figure 4.2 Survival curves for *S. enteritidis* PT4 grown at three pH levels when exposed to 2.5M NaCl

Table 4.4 The tolerance of *S. enteritidis* PT4 previously grown at three pH values to osmotic stress (2.5M NaCl)

Growth pH		D (hours)
4.35	* [73.2 ± 21.6 [n=3]
7.10		48.2 ± 4.8 [n=4]
9.45		1.8 ± 0.2 [n=3]

D = time to kill 90% of the population
values are means ± standard deviation

*shows pair-wise comparisons which are significantly different at the 5% level using Scheffé analysis.

n=sample size

4.3.4 Tolerance to the lethal activity of crystal violet

The resistance of the cultures to the lethal activity of crystal violet was distinct. Acid-grown cells were significantly ($p<0.05$) more resistant to the dye when compared to cells cultivated at pH 7.1 and pH 9.45 (Fig. 4.3 & Table 4.5). Cells grown at alkaline pH were also significantly ($p<0.05$) more sensitive to crystal violet than those grown at neutrality.

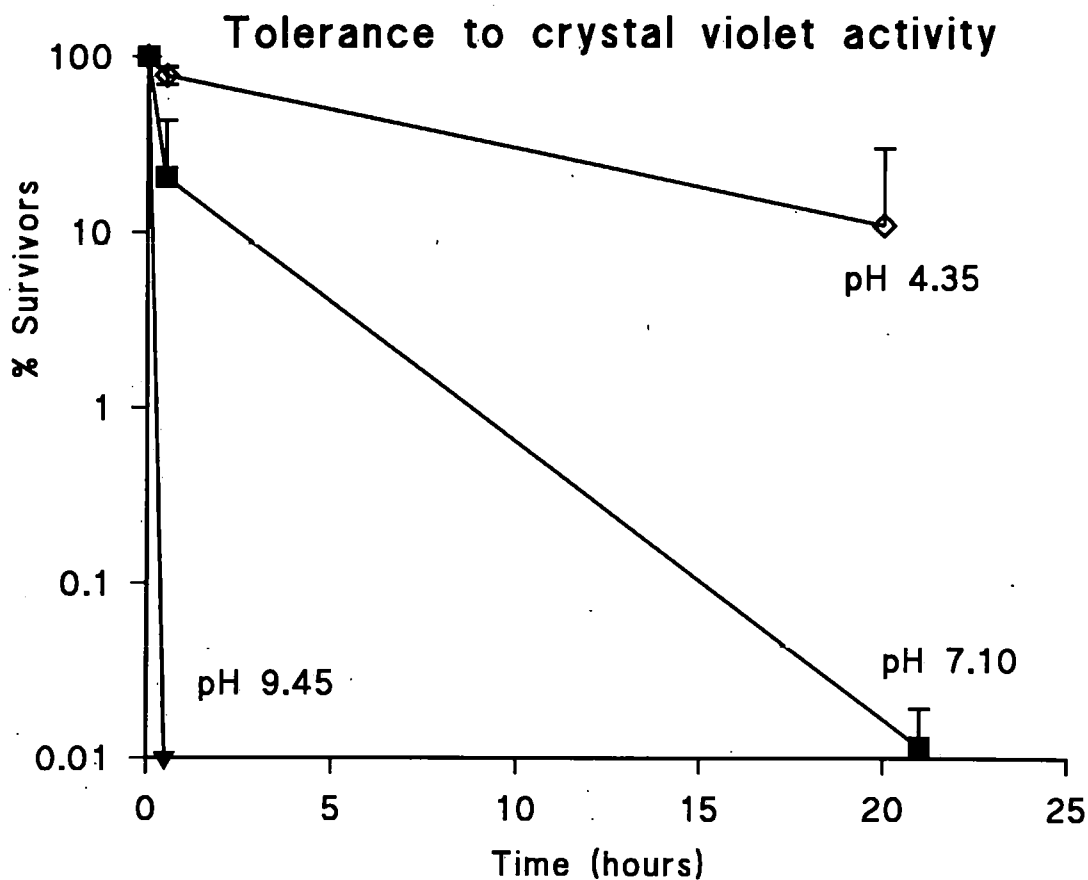


Figure 4.3 Survival curves for *S. enteritidis* grown at three pH values when exposed to the bactericidal activity of crystal violet

Fig. 4.3 illustrates that the tolerance of cells grown at different pH values varied markedly in the presence of 0.0025% (v/v) crystal violet, very few pH 9.45 cells survive for longer than 30 min; whilst the proportion of viable pH 7.10 cells has fallen to about 0.01% after 21 hours, in contrast approximately 10% of pH 4.35 cells survive for 21 hours in crystal violet.

Table 4.5 The tolerance of *S. enteritidis* PT4 grown at different pH levels to the bactericidal activity of crystal violet

Growth pH	D (hours)
4.35	12.1 ± 3.1 [n=3]
7.10	5.9 ± 1.1 [n=4]
9.45	0.1 ± 0.03 [n=3]

D = time to kill 90% of the population

values are means ± standard deviation

*shows pair-wise comparisons which are significantly different at the 5% level using Scheffé analysis.

4.3.5 Summary of the effect of growth pH on the survival of *S. enteritidis* PT4

Average survival times have been determined for a selection of challenge conditions and are shown in Table 4.6. The values have all been quoted in minutes to ease comparison of the different stress factors. The data illustrates that *S. enteritidis* PT4 is able to survive for relatively long periods under a variety of stress conditions, particularly when the cells were grown under acid conditions.

Table 4.6 Average survival times in minutes for *S. enteritidis* PT4

Challenge factor	Average Survival Time (min)		
	Growth pH		
	pH 4.35	pH 7.10	pH 9.45
NaCl	1362	898	35
Crystal violet	226	109	2
50°C (pH 7)	29	8	26
pH 2.0	0.6	0.3	0.3
pH 3.0	69	7	10
pH 10.0	12	9	16
pH 11.0	0.8	0.7	1.1

Table 4.7 A summary of the effect of growth at the limits of the pH range on the tolerance of *S. enteritidis* PT4 when compared to cultures grown at neutrality.

Growth pH	Induced tolerance to	Induced sensitivity to
pH 4.35	low pH high pH 50°C NaCl crystal violet	low temperature
pH 9.45	high pH 50°C	low pH low temperature NaCl crystal violet

4.4 Discussion

S. enteritidis PT4 persists as a frequent cause of foodborne disease (Fig. 1.2). Diverse conditions are found in the food products implicated in outbreaks of disease (Table 1.1), highlighting that *S. enteritidis* PT4 has the ability to survive a range of environmental stresses. In this investigation, the resistance of *S. enteritidis* PT4 cultures grown at different pH levels to extremes of pH, temperature, osmolarity and the lethal activity of crystal violet was compared.

Death affected by environmental factors is often logarithmic in nature i.e. a linear relationship is obtained between log survivors and time. This straight line relationship has been interpreted as indicating that death is the result of

inactivation of only one site per cell, giving a first order reaction relationship (Stephens *et al.* 1994). The survival curves obtained in the present study were not always linear over the entire test period, but tended to be sigmoid in nature (e.g. **Fig. 4.2**). The sigmoid shape suggests some deviation from 'first order' kinetics perhaps due to the non-homogeneity of bacteria within a population, or possibly because some stress factors had multiple actions and consequently did not conform to 'first order' reaction kinetics. The killing action of acid is generally associated with cell membrane damage, although the critical event can also be damage to DNA or enzymes (Booth 1985). The lethal action of high pH to *S. enteritidis* cells has been associated with disruption of the cytoplasmic membrane (Mendonca *et al.* 1994).

An initial 'shoulder' was frequently observed in the plots of log survivors against time before the death rate became exponential. This is probably due to a slight delay before all cells equilibrate to the challenge conditions and also to cell density protection; cells within clumps may have added protection. If the organisms are in groups or clumps, a colony will still develop on the recovery medium until each individual organism is killed, i.e. no decline in viable count will be observed until the entire clump is non-viable. Alternately, or even in conjunction with the previous explanations, the 'shoulder' may represent a period where repair mechanisms are functioning and death occurs only when the rate of damage exceeds that of the repair rate.

In addition to the initial 'shoulder', 'tailing' at the end of the survival curve was observed on some occasions. This final phase can be accounted for by the percentage of the population with increased resistance to the stress factor. Also products from the many killed bacteria may provide protection for the few remaining viable cells.

Cultures grown at different pH levels demonstrated distinct tolerance levels to the test factors (**Tables 4.2 to 4.7**), indicating that pH adaptation induced changes that influenced the subsequent survival potential of the organisms. As the resistance of high and low pH cultures varied, it suggests that the molecular

responses for adaptation to growth at low pH differ, to some degree, from those involved in alkaline growth adaptation.

In general, cells grown at low pH were the most resistant to subsequent stresses. In *S. typhimurium* it has been proposed that acid adaptation induces changes in metabolism which enhance internal pH homeostasis (Foster and Hall 1991). Passive H⁺ conductance and buffering capacity are increased in *S. typhimurium* in response to acidic external pH (Rius *et al.* 1995). In the present study, acid adaptation induced a general protection mechanism in *S. enteritidis* PT4 which provided cross-protection against:- pH, high temperature, osmotic stress and crystal violet activity (Table 4.7). Similar observations have been made with *S. typhimurium*, where acid shock induced significant cross-protection to heat, oxidative stress and osmotic stress (Lee, I.S. *et al.* 1995).

Induction of a general resistance providing cross-protection against a range of factors was previously only associated with starvation stress and stationary phase physiology. In this study, a general stress resistance was observed in *S. enteritidis* PT4 cells during exponential growth under acidic conditions. Bacteria in the stationary phase of growth tend to be more resistant to stress challenges including acid stress (Mossel *et al.* 1995). The induction of cross-protection following acid shock in *S. typhimurium* was shown to be dependent upon the RpoS system (Foster 1995; Foster and Spector 1995); (Table 4.1). In addition to these pH dependent systems, the general stress resistance induced by stationary phase cells is also regulated by sigma S, (Lange and Hengge-Aronis 1991; Hengge-Aronis 1993). Therefore, the RpoS system may also have a role in the general tolerance of exponential phase cells of *S. enteritidis* PT4, adapted to growth at low pH, observed within this investigation.

Increased acid resistance has also been demonstrated in *S. enteritidis* PT4 following short-term exposure (minutes) to pH values in the range pH 3 to pH 6 (Humphrey *et al.* 1993a). Maximum acid resistance was observed following incubation at pH 4 (Humphrey *et al.* 1993a). In the present study, adaptation to growth at extreme pH values rather than 'pH shock' was studied and the

responses to stress challenges were determined using cells in exponential growth phase. Cells grown at pH 4.35 had a D_{90} -value of 18 ± 9 min at pH 2.5 (Table 4.3); this is longer than the published value for *S. enteritidis* PT4 cells habituated for 30 min at pH 4 where the D_{90} -value was 6.2 ± 1.1 min (Humphrey *et al.* 1993a). It is not possible to determine whether the levels of acid resistance induced by growth at low pH are different from those following short-term acid habituation. Incubation temperatures, nutrient availability, level of available oxygen and bacterial strains varied between the two studies, all of which have been shown to influence the survival capabilities of bacteria. Consequently, all these variables may mask any differences attributable to the nature of the adaptation process. However, pH adaptation following growth at hostile pH levels may be expected to involve both genotypic and phenotypic responses, whilst short-term acid shock would allow time for only phenotypic responses.

Acid adaptation also gave some cross-protection against high pH; acid-adapted cells survived slightly longer at pH 10 and pH 11 when compared to those grown around neutrality (Tables 4.3 and 4.6). Cells adapted to low pH were able to survive, on average, for about 12 min at pH 10, (equivalent D_{90} = 37.6 min) and for ~0.8 min even at pH 11 (D_{90} = 2.5 min) (Table 4.6). However, acid adapted cells were not as resistant to high pH values as cells previously grown at alkaline pH (Table 4.3).

In contrast, growth at alkaline pH induced sensitivity to acid, osmotic stress and crystal violet activity when compared to cultures grown at pH 7.10 or pH 4.35. The most dramatic changes were in the tolerance to crystal violet and osmotic stress where the survival times were significantly ($p < 0.05$) shorter than those for pH 7.10 or pH 4.35 cells. The average survival times were reduced from approximately 105 and 900 min, respectively, for pH 7 cells to 1.9 and 345 min, respectively, for pH 9 cells (Tables 4.6 and 4.7). This indicates that alkaline adaptation may predispose cells to subsequent damage by altering the surface of the bacteria. Acid sensitivity has also been demonstrated in *E. coli* cells raised to pH 9.0 (Rowbury *et al.* 1993a, b). When *Enterococcus faecalis* was subjected to alkaline shock at pH 10.5 similar observations to this investigation were made;

the resistance to high pH was increased simultaneously with increased acid sensitivity (Flahaut *et al.* 1997).

S. enteritidis PT4 cells grown at pH 9.45 demonstrated significantly ($p < 0.05$) enhanced thermo-tolerance when compared to those grown at neutrality. The levels of heat resistance demonstrated by acid and alkali cells were similar and were both significantly ($p < 0.05$) higher than those of pH 7 cells. The average survival times for acid and alkaline grown cells were 29 and 26 min, respectively, when the 50°C challenge was performed at pH 7 (the equivalent D-values were 91 and 85 min, respectively). In contrast, the average survival time of cells grown at pH 7 was only 23 min (Fig. 4.1, Tables 4.2 and 4.6). Other workers have also reported increased resistance to 55°C in cultures of *S. enteritidis* PT4 following short-term (a minimum of 5 min) exposure to pH 9.2 when the heat challenge was carried out at pH 7 (Humphrey *et al.* 1991a). When the heat challenge was performed at pH 9, a similar increase in heat resistance was not observed (Humphrey *et al.* 1991a).

The effect of pH during the thermal challenge was also investigated. Cultures were challenged both at the original growth pH and also at pH 7. *S. enteritidis* PT4 cells survived significantly ($p < 0.05$) longer under adverse temperatures when the pH was at neutrality, irrespective of the previous growth pH (Tables 4.2 and 4.6). This illustrates that extreme temperatures and pH levels have an additive inhibitory effect. Although adaptation to a number of stresses or starvation increases thermal tolerance in salmonellae by a mechanism involving heat shock proteins, the synthesis of heat shock proteins has not been shown to enhance acid resistance (Leyer and Johnson 1992). This indicates that the stress proteins induced by pH adaptation are not solely 'heat shock' responsive but that they do stimulate protection mechanisms which give cross-protection against thermal stress.

Strains with enhanced thermo-tolerance have been shown to survive longer on surfaces, (Humphrey *et al.* 1995). This will be an important factor in the persistence of the organism in the environment; the longer cells remain viable on

surfaces, the greater are their chances of encountering more favourable conditions by cross-contamination into new environments.

Cells grown at the extremes of pH declined in numbers when exposed to 4°C, whereas no decrease in cell viability was detected during the test period in cells grown at pH 7.10. The mechanisms required to survive low temperatures appear to differ from those involved in high temperature survival; growth at the extremes of pH induced low temperature sensitivity at the same time as increasing the resistance to high temperature (Tables 4.2, 4.6 and 4.7).

Contrasting levels of tolerance to osmotic pressure and crystal violet activity were displayed by populations grown at the pH limits; increased resistance followed growth at low pH whilst sensitivity was induced by alkaline pH (Tables 4.6 and 4.7). This may be a consequence of different changes in cell surface properties following adaptation to the two pH levels. The cell surface has been shown to perform a major role in detection and adaptation processes, and in pathogenesis e.g. adaptation of *S. typhimurium* to intracellular growth involves structural alterations to the peptidoglycan (Quintela *et al.* 1997).

Crystal violet resistance was determined at pH 7 as the external pH determines the degree of ionisation of the dye and subsequently the level of lethal activity (Adams 1967). The negative charge on the bacteria increases as the pH of the medium rises, and the organisms become more sensitive to the dye. This was demonstrated with *S. anatum* where cells at pH 5 could tolerate approximately 100 fold higher concentrations of crystal violet than cells at pH 9 (Moats and Maddox 1978). Crystal violet tolerance varies amongst clinical isolates and it has been speculated that the tolerance levels may be reflective of the previous growth conditions of the organisms. Consequently, it has been postulated that crystal violet tolerance could possibly be used as a tool in epidemiological studies (Carraminana *et al.* 1997).

In summary, gene regulation in response to acidic pH induced cross-protection against pH, heat, salt and crystal violet activity, demonstrating that acid adaptation is potentially an important mechanism in the survival of *S. enteritidis* PT4. The global protection response induced by acid conditions will enhance the survival of *S. enteritidis* PT4 during food processing procedures and during infection of a host. For example, it has been reported that acid-adapted cells of *S. typhimurium* demonstrated increased resistance to inactivation by organic acids in cheese (Leyer and Johnson 1992).

Generally, non-pH adapted cells were less tolerant of challenges of pH and high temperature (Tables 4.2, 4.3 and 4.6). However, cultures grown continuously at pH 7.10 were the most resistant to low temperature; indeed, cells grown at pH 7.10 initially continued to increase in numbers at 4°C (Table 4.2). The consequences of adaptation to high pH levels varies, with enhanced resistance to some stresses being induced at the same time as increased sensitivity to others (Tables 4.6 and 4.7).

The data obtained demonstrate that the growth environment dictates the physiology of the cells; this was also shown in Chapter 3, (McDermid *et al.* 1996). The physiological state of an organism clearly has a significant role in its survival in foods, the environment and during infection. The resistance of acid- and alkaline-adapted cells differed, particularly to the surface active agents NaCl and crystal violet (Tables 4.6 and 4.7). This implies that the cell surface responses of *S. enteritidis* may vary dependent upon whether the external environment is acidic or alkaline.

Comparisons of the acid survival strategies of *S. typhimurium*, *E. coli*, and *Shigella flexneri* demonstrated that although the species are closely related their survival strategies are dramatically different. It was speculated that these differences were reflective of the different needs of the bacteria in their ecological niches (Lin *et al.* 1995), illustrating that the acid response may have evolved to play a major role in pathogenesis.

Further studies of the pH adaptation mechanisms will follow:- the effect of pH adaptation on the virulence of *S. enteritidis* PT4 will be investigated in **Chapter 5** and the modulation of protein expression in response to growth pH will be addressed in **Chapter 6**.

Chapter 5

The Effect of Environmental Growth pH on the Virulence of *S. enteritidis* PT4 Strain 226 405

5.1 Introduction

Gastrointestinal diseases are a problem in the developing areas of the world and also in industrialised regions, despite greater emphasis on measures to improve the microbiological quality of food in these areas. The rise in *S. enteritidis* infections in humans has been partially attributed to the consumption of eggs and poultry products from flocks with an increased prevalence of *S. enteritidis* (Section 1.5). Other contributing factors could be:- increased virulence of the prevalent strains *per se*, or as a consequence of adaptation to conditions associated with frequently implicated food products inducing responses which enhance pathogenicity. The reasons why *S. enteritidis* PT4 is better able to cause infection than other bacteria which commonly contaminate food products, e.g. *S. binza* and *S. derby*, are still to be determined.

S. enteritidis frequently encounters potentially lethal environmental stresses; these include pH values from mild to extreme, both above and below the range for growth. pH extremes are found in food products, host and natural environments and possibly even as a consequence of their own metabolism. An increased ability to survive pH stress will widen the range of ecological niches in which *S. enteritidis* can persist and this will enhance their pathogenicity.

5.1.1 Host environments

A range of host defence mechanisms determine whether clinical manifestations follow interactions between *S. enteritidis* and the host. pH plays a non-specific role in the host defences at several sites, therefore, the physiological adaptation of bacteria to pH extremes will have a significant effect on their fitness to persist within the host. The stomach is the first barrier where pH has a major inhibitory role, with the pH of the empty stomach in man being in the region of pH 2

(Madara 1991). The number of viable bacteria declines rapidly in response to the lowering pH as ingested food stimulates acid secretion within the stomach (Drasar *et al.* 1969). However, food can have a buffering capacity, the level of which is dependent upon the food type (Mackie *et al.* 1997), which can provide some protection for the bacteria within the food *milieu* by maintaining the pH above pH 4. This not only allows bacteria to survive passage through the gastric barrier, but will also allow metabolic activity and possibly growth (Milton-Thompson *et al.* 1982), **Chapter 4**. Mildly acidic stress within food in the stomach may elicit pH shock responses by *S. enteritidis*.

The number and type of bacteria which persist in the upper gastrointestinal tract is predominantly controlled by the age and diet of the host, the pH, the flow through the site and the complexity of the interactions between microbes and with the host. It has been estimated that the number of microbial cells inhabiting the human body exceeds the number of human cells that make up the host *i.e.* 10^{14} versus 10^{13} (Luckey 1972). Consequently, the ability to outcompete other microbes will contribute to the colonisation success of *S. enteritidis*. The pH of the sites that *S. enteritidis* colonises will be largely dependent upon the metabolic products of the resident bacteria.

The ability of the commensal flora to exclude invading bacteria has been referred to as colonisation resistance or antagonism (Tannock 1984). Colonisation resistance was clearly demonstrated, when the infective dose of *S. enteritidis* for rodents was reduced following disruption of the commensal gut flora with streptomycin (Bohnhoff *et al.* 1954; Miller, C.P. and Bohnhoff 1963). The normal gut flora was thought to inhibit the colonisation of salmonellae by the production of volatile fatty acids (VFAs) (Meynell and Subbaiah 1963; Bohnhoff *et al.* 1964; Que *et al.* 1986).

The levels of VFAs present in the normal mouse caecum are sufficient to prevent the multiplication of *S. enteritidis in vitro*, with the inhibitory activity being greatest at low pH and low Eh levels. Above pH 7, VFAs are primarily in the dissociated form and unable to inhibit the growth of enteric pathogens (Hentges 1983). As

the pH falls the proportion of undissociated acid increases, enabling their entry into bacterial cells with subsequent inhibition of metabolism. Once inside the cell, both dissociated and undissociated acids can interfere with metabolism. Fatty acids inhibit bacterial growth by uncoupling oxidative-phosphorylation.

Environmental pH influences the affinity (K_s) of bacteria for the available nutrients, and consequently the maximum growth rate, (μ_{\max}), (Section 3.2.3), which in turn will influence the ability of the bacteria to outcompete others. The rate of flow of material through the lumen of the gastrointestinal tract particularly in the upper and middle regions of the small intestine is an important factor in the persistence of bacteria. If the rate of flow is greater than μ_{\max} under the prevailing conditions then the bacteria will be passed through the system unless they are able to attach. Passage through the colon is generally slow enough not to exceed μ_{\max} values.

Infecting *S. enteritidis* organisms also experience varying pH levels when they encounter host phagocytic cells. As a result of phagocytosis, micro-organisms become enclosed in vacuoles (phagosomes) within the cytoplasm of the phagocytic cell. At this stage the pH is alkaline which enables the cationic proteins, which have antibacterial action, to bind to the bacteria (Segal *et al.* 1981). Lysosomal granules move to fuse with the membrane of the phagosome forming phagolysosomes and then discharge their contents into the vacuole, lowering the pH to 3.5-4.0. The significance of pH during phagocytosis in the killing of *S. enteritidis* is unknown as pH represents only one component of the killing arsenal.

5.1.2 pH levels associated with implicated foods

Although a wide range of foods have been implicated in salmonellosis, the majority of cases result from the consumption of meat, poultry, seafood or other products of animal origin such as unpasteurized egg products and unpasteurized milk and dairy products (Ryan *et al.* 1996). A wide variety of environmental conditions prevail with these foods (Section 1.11).

Where it was possible to determine the site of contamination within eggs the albumin was found to be more frequently contaminated than the yolk (Humphrey *et al.* 1991b). *S. enteritidis* can persist and grow within albumin (Clay and Board 1991; Humphrey and Whitehead 1992; Lock and Board 1992), even though the pH rises to about pH 9.5 within a few days of laying (Board *et al.* 1994). High pH is also experienced by *S. enteritidis* cells which contaminate the egg shell during the washing process (Holley and Proulx 1986). Occasionally cells of *S. enteritidis* survive this washing especially when the alkaline pH of the wash water falls towards pH 10 (Jones, F.T. *et al.* 1995).

In contrast, in mayonnaise which has repeatedly acted as a vehicle in outbreaks (Perales and Garcia 1990; Ahmed *et al.* 1992; Irwin *et al.* 1993) *S. enteritidis* has to tolerate acidic conditions (~pH 4) (Smittle 1977). Acid adaptation has also been shown to be important for the survival of *S. typhimurium* in fermented dairy products such as cheeses (Leyer and Johnson 1992). The pH of meat products is often around neutral completing the range of pH levels experienced by food-contaminating organisms.

5.1.3 Virulence factors

Virulence can be defined as the ability to cause disease, the best measures of which are morbidity and mortality. To be a successful pathogen does not imply that the organism must kill the host (this would reduce the number of host species), as survival and replication are the key objectives. It could be considered that the most successful pathogens are those which require a small number of organisms to initiate infection, have the ability to colonise multiple sites within the host, are able to survive intact the challenges of the immune response, and leave the host in a condition which would assist transmission to a subsequent host or non-hostile environment. Many varied properties are required by *S. enteritidis* to meet these objectives, the molecular components of which can be defined as virulence factors.

In general, virulence factors have specialised functions and include:- the RpoS transcriptional factor, motility, adhesins, invasins, lipopolysaccharides and proteases, all of which are not necessarily used by a single pathogen, and the

importance of each varies with the stage of infection (Jones, B.D. and Falkow 1996). The classification of virulence factors is obviously complex as some factors contribute to both 'house-keeping' functions and pathogenicity e.g. iron chelators which are required for growth during infection and in any environmental situation where iron levels are low, such as in fresh albumin. At least 60 genes have been identified as required for virulence in mice using several serovars of *Salmonella* including *S. enteritidis*, *S. typhimurium*, *S. choleraesuis* and *S. dublin* (Groisman and Ochman 1997).

5.1.3.1 Adhesins

The gastrointestinal tract provides a large surface area along which intimate contact between a foodborne pathogen such as *S. enteritidis* and the host can occur. Attachment to the epithelial surface provides several advantages:-

- it prevents bacteria being cleared by the flow of the gastrointestinal contents,
- it increases the chances of the bacteria obtaining potential nutrients that leak from the epithelia,
- the pH at the epithelial surfaces is generally around neutrality providing a more suitable environment than within the contents of the digestive tract,
- higher oxygen levels - the properties of bacteria associated with the mucosal surfaces of rodents suggest microaerophilic conditions, and this could be a consequence of oxygen diffusion from the epithelia (Roach and Tannock 1979; Phillips and Lee 1983),
- stable association with the epithelium is a prerequisite for the subsequent stage of infection, i.e. invasion.

The potential role of fimbriae as adhesins has long been recognised (Duguid *et al.* 1966), although their functions in the pathogenesis of *S. enteritidis* remain unclear (Thorns 1995). The picture is complicated by the number of surface fimbrial antigens expressed by *S. enteritidis* (**Section 3.1.2**), (Thorns *et al.* 1996). There is some evidence that their role may involve mediation of a close stable interaction between host and bacterial cell surfaces prior to invasion (Grund and Stolpe 1992; Satta *et al.* 1993; Peralta *et al.* 1994).

Adhesins also contribute to interactions between bacteria during the formation of micro-colonies which are required for invasion; a minimum number of bacteria may be required for some events during infection (Dorman 1994).

In addition to the pilus adhesins, bacteria can also express surface proteins which act as adhesins; these are usually outer membrane proteins in Gram negative species. Cell surface hydrophobicity may also have a role in attachment to epithelial cells (Mackie *et al.* 1997). The details of the mechanisms utilised by *S. enteritidis* for attachment are still to be determined.

5.1.3.2 Invasion mechanisms

The epithelial cells of the gastrointestinal tract must facilitate the absorption of nutrients and water whilst acting as a barrier to enteric pathogens. The epithelium acts both as a physical and an immunological barrier with approximately one quarter of the intestinal mucosa being lymphoid tissue. In fact, more than seventy percent of all immune cells are located within the intestines (Kraehenbuhl and Neutra 1992; Kagnoff 1993).

Penetration of the intestinal mucosa is a prerequisite for systemic infection and is also thought to be essential for the induction of diarrhoea. Salmonellae invade various epithelial cells along the gut. It has been shown that *S. typhimurium* specifically targets M cells to cross the intestinal barrier (Jones, B. *et al.* 1995). M cells have unique features which facilitate bacterial penetration; these include: low levels of mucus and brush border enzymes such as alkaline phosphatase, few lysosomes but have a well organised microvascular system (Kato and Owen 1994).

The signalling pathway and mechanisms used during internalisation of *S. enteritidis* remain unclear. Genetic studies suggest that they are complex involving several chromosomal loci for maximum invasion (Stone *et al.* 1992).

Environmental regulation of invasion genes has been demonstrated; for example, oxygen limitation increased invasiveness of *S. typhimurium* (Ernst *et al.* 1990),

and high osmolarity induced changes in the levels of DNA supercoiling which affected the expression of invasion genes (Galan 1996).

5.1.3.3 Flagella and motility

The structure and function of the flagellum (H antigen) (Section 3.1.1) has been characterised as a virulence factor, although, their specific activities in virulence remain unclear. The high frequency of phase and antigenic variation in flagellins from clinical isolates of *S. typhimurium* suggests an important role for flagella in evading the host responses; in addition, a role for flagella in adherence to host cells has been postulated. Motility and chemotaxis enable bacteria to reach sites more suitable for colonisation (Harshey and Toguchi 1996).

Motility has been shown to enhance virulence in some studies using *S. typhi* (Grossman *et al.* 1995) and *S. typhimurium* (Lee, M.D. *et al.* 1996), although, mutants of *S. typhimurium* lacking flagella or motility have also been shown to remain virulent (Lockman and Curtiss, R. III. 1990). Furthermore, chemotaxis and motility mutants demonstrate altered invasive properties (Betts and Finlay 1992; Jones, B.D. *et al.* 1992). Thus the role of flagella in the pathogenesis of *S. enteritidis* is still to be elucidated.

5.1.3.4 Virulence plasmids

Plasmid carriage by *S. enteritidis* was described in Section 3.1.3. The role of the virulence plasmids remains unclear, since plasmid-free strains of *Salmonella* have been implicated in outbreaks of enteritis in humans (Kapperud *et al.* 1990; Threlfall *et al.* 1994), and plasmid-cured strains maintain virulence and invasiveness in chickens (Halavatkar and Barrow 1993). It has become accepted that the plasmid genes are not required for translocation of the intestinal mucosa (Manning *et al.* 1986; Pardon *et al.* 1986; Heffernan *et al.* 1987) but are associated with intracellular growth (Gulig and Doyle 1993) and accumulation of bacteria in the liver and spleen.

5.1.3.5 Genetic diversity

The genome of respiratory pathogens is smaller than that of enteric pathogens. This is thought to be due to gut pathogens acquiring genetic information from the

vast number of bacteria and viruses they have close association with in the gastrointestinal tract. Acquiring information which enhances pathogenicity or survival could enhance the persistence of enteric pathogens.

The dynamic nature of a transcriptionally-active genome allows for enhanced genetic diversity within a bacterial population. It is possible that some members of a bacterial community express genetic information whilst others store the information in a cryptic state. Maintaining low percentages of the population with different phenotypes reduces the risk of elimination. If the number of bacteria expressing disadvantageous genes is low at any one time their loss would be irrelevant to the population.

5.1.3.6 Evasion of the host defences

A vital part of the infection process involves avoiding destruction by the arsenal of host defences. *Salmonellae* are considered to be intracellular pathogens as they can survive and even replicate within the intracellular environment of a variety of eukaryotic cells including phagocytic cells (Leung and Finlay 1991). There is general agreement that *Salmonella* species stay within a membrane-bound vacuole both in phagocytic and non-phagocytic cells. Research is now directed towards identifying the virulence factors which enable the bacteria to thrive within these vacuoles (Jones, B.D. and Falkow 1996).

5.1.3.7 Tolerance

A wide range of environmental conditions persist during food production and within a host. Clearly, an increased ability to survive extreme environmental factors will enhance persistence of the bacteria during all the stages of food processing and during infection.

5.1.4 Environmental regulation of virulence factors

A common aspect of pathogenesis is the ability of bacteria to vary the expression of molecules or structures which are involved in interactions with the host. There is overwhelming evidence that some if not all virulence determinants are under environmental regulation (Griffiths 1991), and that different virulence determinants are frequently controlled by common regulatory systems.

Environmental signals such as pH, temperature, osmolarity, oxygen and CO₂ levels, and availability of ions, may be used by the bacteria to induce appropriate responses to the changing host conditions (Hall and Foster 1996).

5.1.5 Virulence models

Both clinical symptoms and immune responses can be used as markers of virulence. The selection of the model system for studying the virulence of *S. enteritidis* is of crucial importance as extrapolation between animal species is not always valid, and virulence is multifactorial; the most relevant experimental model would be the natural host. The mouse has become the standard model for studying systemic typhoid infections; however, many different models are routinely used to study different aspects of *S. enteritidis* PT4 infection, some examples of which are described below.

Rats have been the favoured model for studies involving endotoxins from *S. enteritidis* (Goto *et al.* 1990; Zeller *et al.* 1991). The rat model has also been used to follow the course of infection with *S. enteritidis* and *S. typhimurium* (Naughton *et al.* 1995).

One-day-old chicks and laying hens are the most appropriate models for investigating disease in poultry. These models have also been used in experiments to obtain fundamental information about the virulence of *S. enteritidis*, including studies concerning vaccine protection (Cooper *et al.* 1993; Peralta *et al.* 1994); prediction of egg infection frequency (Gast and Beard 1992); the role of the fimbrial antigen, SEF14, (Thorns *et al.* 1996); invasion (Hinton *et al.* 1990); faecal shedding (Nakamura *et al.* 1993); the role of the 38 MDa plasmid (Halavatkar and Barrow 1993), and *in vivo* expression of phenotypic characteristics (Chart *et al.* 1993).

Similar aspects of the virulence of *S. enteritidis* have also been studied using murine models. BALB/c and C57/BL are amongst the most frequently cited mouse strains, with the oral and intra-peritoneal routes of challenge being commonly used. These studies include:- the effect of passive immunisation with hen egg-yolk antibodies against *S. enteritidis* (Peralta *et al.* 1994); studies of

colonisation resistance against salmonellae (Voravuthikunchai and Lee 1987); the protective responses to temperature-sensitive mutants of *S. enteritidis* (Gherardi *et al.* 1993), identifying vaccine candidates (e.g. lipopolysaccharides and outer membrane proteins) against *S. enteritidis* infection (Chart and Rowe 1991); and comparisons of the virulence of isolates from differing geographical locations (Cox and Woolcock 1994).

Tissue culture cell lines have been used to investigate the invasion properties of *S. enteritidis* (Stone *et al.* 1992). The main limitation of tissue culture techniques is the absence of any host response, making them more appropriate to studies of specific virulence determinants e.g. those related to attachment and invasion.

A murine model was selected in this study as it has several advantages, including the ease of handling these animals, the reduced financial implications of housing these animals, and the relevance of the model to the disease.

5.1.6 Aims

The aim of this study was to determine the influence of environmental pH on the virulence of *S. enteritidis* PT4. *S. enteritidis* frequently enters the host following exposure to extremes of pH (either high or low), which can modulate the growth, physiology, plasmid carriage and survival mechanisms of the challenging organisms (**Chapters 3 and 4**). These factors have all been characterised as virulence determinants and, consequently, will determine the virulence of the organism. Therefore, the specific aims of this investigation were to:-

1. Compare the virulence of *S. enteritidis* PT4 grown continuously at the pH limits for growth, and at neutrality, using a mouse model in terms of both clinical manifestations (mortalities) and tissue invasion.
2. Compare the physiology of the infecting bacteria with those retrieved from the host animal, and
3. Observe tissue samples using electron microscopy.

5.2 Methods

5.2.1 Bacteria and growth conditions

Strain 226 405, a clinical isolate of *S. enteritidis* PT4, was grown in Vogel Bonner medium (Vogel and Bonner 1956) supplemented with vitamins and 2.5 g l⁻¹ hog gastric mucin in a chemostat at pH 4.35, pH 7.10 and pH 9.45 at a dilution rate of 0.1 h⁻¹ (equivalent to a mean generation time of 6.9h), under an atmosphere of 5% CO₂ and 95% N₂, as described previously (Section 3.2.2).

Static batch cultures of *S. enteritidis* PT4 strain 226 405 grown in nutrient broth at 37°C in air were used to make an initial evaluation of the mouse model and for subsequent comparative purposes.

5.2.2 Mouse model

The virulence of cells grown at different pH levels was compared using female C57BL mice weighing approximately 20g. Mice were housed in individual boxes to exclude cross-infection, (animals being reinfected with unknown doses via differing routes), which would confound the experimental protocol and influence the prevalence and course of infection. Food and water were provided *ad libitum* to reduce stress to the animals. Stress has been shown to induce changes within carrier animals which allow salmonellae to multiply throughout the gastrointestinal tract (Tannock and Smith 1972). The mice were fed on a non-irradiated *Salmonella*-free commercial diet (Bond K Universal, Hull).

5.2.3 Animal challenge

C57BL mice were infected either orally or intraperitoneally (i.p.). Oral inoculation was performed by intubating 0.1ml of culture using a ball-end needle 1cm into the oesophagus, and i.p. inoculation was by injecting 0.1ml of culture into the peritoneal cavity. Samples of culture were removed from the chemostat immediately prior to use; any dilutions required to obtain the appropriate dose were made using saline preheated to 37°C to avoid temperature shock.

5.2.4 Virulence assessment

Virulence was determined in terms of the rate and number of post-inoculation mortalities and the levels of tissue invasion. Following challenge, mice were observed twice daily for signs of illness and mortalities recorded with time. Animals exhibiting symptoms of lethargy, hunched gait, ruffled fur and white rings around the eyes were killed by carbon dioxide asphyxia; these symptoms have been shown to be markers of tissue invasion and of animals which would subsequently die. The level of tissue invasion was determined in moribund mice and in any survivors after the 14 day test period.

5.2.5 Tissue invasion

The level of bacterial carriage and the histology of spleen tissue was investigated in sacrificed moribund mice and in survivors at day 14 post infection. The whole spleen was removed aseptically into a sterile preweighed pot and the mass of the spleen determined, a small portion of spleen tissue was then viewed using electron microscopy. The remaining spleen tissue was macerated by passage through a syringe into 0.5ml of saline and then subjected to microbiological analysis. Serial dilutions of the macerates were performed in maximum recovery medium and 0.1ml volumes of the dilutions spread over blood agar plates, which were incubated at 37°C in air for 48 hours prior to estimating the number of colony forming units. Comparisons of the number of intracellular bacteria with those present on the surface of the spleen were made by comparing the number of bacteria obtained by washing the tissue with those obtained after maceration, which caused disruption of the spleen cells.

Enumeration of *S. enteritidis* levels in the liver, blood and caecum content were also carried out in a similar manner; a selective medium (XLD) was used for caecum content samples.

5.2.6 Control animals

Groups of three control animals were either uninoculated, or received 0.1ml of culture filtrate. Filtrates were prepared from cultures at each pH by centrifuging the culture for 10 min at 3,000g, followed by filtration of the supernatant (0.2µm pore size filter, Sartorius, Göttingen, Germany).

5.2.7 Electron microscopy of a spleen sample

A sample of spleen tissue was fixed in 2.5% (v/v) glutaraldehyde, post fixed in 1% osmium tetroxide and embedded in araldite resin using standard procedures. Thin sections were prepared and stained with uranyl acetate and lead citrate prior to examination by transmission electron microscopy.

5.2.8 Data analysis

Comparisons were made using independent chemostat cultures. For statistical analysis, the data from repeat chemostat runs was combined and analysed for significant differences using the large sample test (Fisher and Van Belle 1993):

5.3 Results

5.3.1 Effect of dose and route of challenge

Administration of batch grown cells of *S. enteritidis* PT4 strain 226 405 via intragastric intubation to C57BL mice resulted in only one mortality using doses in the range 10^4 - 10^7 cfu (Table 5.1); whereas 9 out of 10 mice died following intra peritoneal (i.p.) inoculation with 10^5 cfu (Table 5.2). Although the oral route of challenge is more relevant to the modelling of food poisoning, it is also less sensitive and less reproducible than inoculation into the peritoneal cavity. This makes the i.p. route more appropriate for comparative studies as the inherent variation within the technique is reduced. Infection following i.p. challenge of mice provided a basic test for comparing the virulence of *S. enteritidis* PT4 grown at different pH levels. An effect of the dose size was observed with batch grown cells (Table 5.2) and with chemostat cells grown at pH 4.35, however, the proportion of mortalities following challenge with 10^5 or 10^6 cfu of pH 9.45 cells was similar (Table 5.3). A dose of 10^6 cfu was selected for use in the comparative studies as this exceeded the infective dose for all growth conditions tested.

Table 5.1 The effect of infective dose following oral challenge of C57 mice with *S. enteritidis* PT4 226 405 batch grown in nutrient broth

Dose (cfu)	Number of deaths/ number of mice challenged
10^4	0/10
10^5	0/10
10^6	1/10
10^7	0/10

Table 5.2 The number of deaths with time post i.p. challenge with batch grown *S. enteritidis* PT4

Dose (cfu)	Days post infection										Total deaths /no. mice
	1	2	4	5	6	7	8	9	12	14	
10^3	0	0	0	0	0	0	0	0	0	0	0/10
10^5	0	0	0	0	5	6	7	9	9	9	9/10

Table 5.3 The effect of dose size on the number of deaths with time after i.p. inoculation with chemostat cells grown at pH 4.35 or pH 9.45

pH	Dose cfu	Days post infection										Deaths/no. of mice
		1	2	3	4	5	6	7	10	11	14	
4.35	6×10^4	-	-	-	-	-	-	2	-	1	4	4/10
	7×10^6	1	-	8	-	-	-	-	-	-	-	9/10
9.45	1×10^5	-	-	2	-	-	1	-	1	-	-	4/10
	5×10^6	1	-	-	-	1	-	-	-	1	1	4/8

5.3.2 Effect of growth pH on the virulence of *S. enteritidis* PT4

The number of deaths with time after i.p. administration of approximately 10^6 cfu of *S. enteritidis* PT4, previously grown at a range of pH values was recorded (Table 5.4; Fig. 5.1). pH 4.35 cells were the most virulent, both in terms of the number and rate of mortalities. The number of mortalities in mice challenged with pH 4.35 cells was significantly higher than in those inoculated with pH 9.45 cells ($z=1.74$, $p=0.04$) and approached being significantly higher when compared to pH 7.10 cells ($z=1.57$, $p=0.06$) 14 days post challenge. There was no significant difference in the number of mortalities following challenge with pH 9.45 cells when compared to pH 7.10 cells, ($z=0.06$, $p= 0.48$) at day 14. The rate at which the cultures induced disease varied, at days 3 and 4 the proportion of mortalities was significantly different for each pH culture ($p<0.01$). Significantly more mortalities occurred with pH 4 cells than with pH 7 or pH 9 cells and the proportion of mortalities with pH 9 cells was significantly higher than with pH 7 cells. No *S. enteritidis* cells or disease symptoms were observed in the control animals.

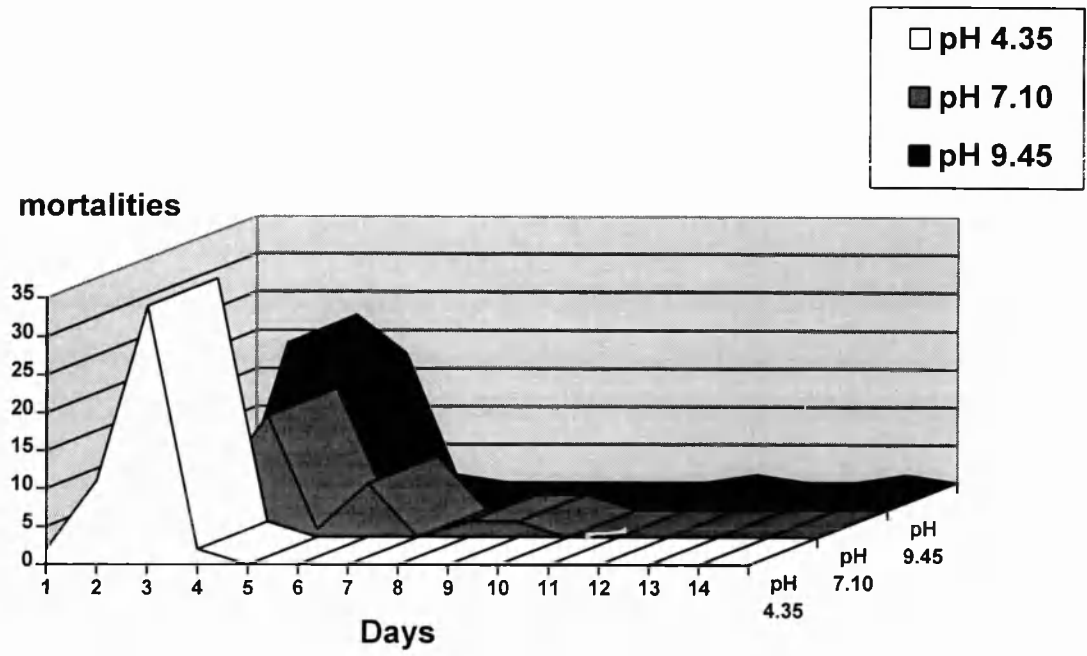


Figure 5.1 The virulence of *S. enteritidis* PT4 grown at different pH values

Table 5.4 The number of deaths with time after i.p. inoculation with *S. enteritidis* PT4 previously grown at a range of pH values

pH	Days post infection										Deaths /no. of mice	Dose cfu
	1	2	3	4	5	6	8	9	11	14		
4.35	-	8	10	2	-	-	-	-	-	-	20/20	2x10 ⁶
	1	3	16	-	-	-	-	-	-	-	20/20	2x10 ⁶
	1	-	8	-	-	-	-	-	-	-	9/10	7x10 ⁶
SUM	2	11	34	2	-	-	-	-	-	-	49/50	
7.10	-	1	6	12	-	-	1	-	-	-	20/20	5x10 ⁶
	-	-	1	4	1	1	-	-	-	-	7/10	6x10 ⁶
	-	-	-	-	-	6	1	2	-	-	9/10	7x10 ⁶
SUM	-	1	7	16	1	7	2	2	-	-	36/40	
9.45	-	-	3	16	-	-	-	-	-	-	19/20	2x10 ⁶
	-	-	18	1	1	-	-	-	-	-	20/20	2x10 ⁶
	-	1	1	-	-	-	-	-	1	1	4/8	5x10 ⁶
SUM	-	1	22	17	1	-	-	-	1	1	43/48	

5.3.3 Invasiveness of *S. enteritidis* PT4 grown at different pH values

Similar levels of bacterial carriage were observed in spleen samples from all moribund animals irrespective of the growth pH of the infecting bacteria; approximately 10⁸ *S. enteritidis* PT4 cfu ml⁻¹ of macerate were isolated. The levels were significantly lower in surviving animals, where approximately 10⁴ cfu ml⁻¹ of macerate were recovered (**Table 5.5**). The number of bacteria recovered was always greater (by a factor of several logs) when the spleen cells were disrupted prior to determining the level of bacterial carriage (**Fig. 5.2**). This indicates that a large proportion of the *S. enteritidis* PT4 cells are within the spleen tissue and not just contaminating the surfaces.

Enlarged spleens were observed in moribund animals, with the mass of some organs having increased by approximately 3 fold (**Table 5.6**). Between 10-50% of cells re-isolated from the spleen macerates of moribund animals previously infected with pH 4.35 cells were motile, compared with only 1% of the challenging population, when estimated by light microscopy (**Table 3.2**). These results were confirmed by EM where approximately 17% of the re-isolated population expressed flagella, compared to only 2% of the challenging cells (**Table 3.2**). The proportion of the re-isolated population which were expressing fimbriae could not be determined using EM due to the presence of contaminating spleen cell debris.

Table 5.5 Spleen carriage of *S. enteritidis* grown at different pH levels in moribund and surviving animals

Growth pH of cells	Dose (log ₁₀ cfu)	Health status	Spleen macerate (log ₁₀ cfu.g ⁻¹)
4.35	6.8	moribund	8.3
7.10	6.0	moribund	7.4
9.45	6.7	moribund	7.9
7.10	6.0	survivor	4.7
9.45	6.7	survivor	4.6

Table 5.6 The weight of spleen organs from moribund and control animals

Group of Animals	Mean spleen mass (g)
Control	0.09 ± 0.02 [6]
Moribund	0.32 ± 0.08 [9]

values quoted as means ± standard deviations

sample size is shown in parenthesis

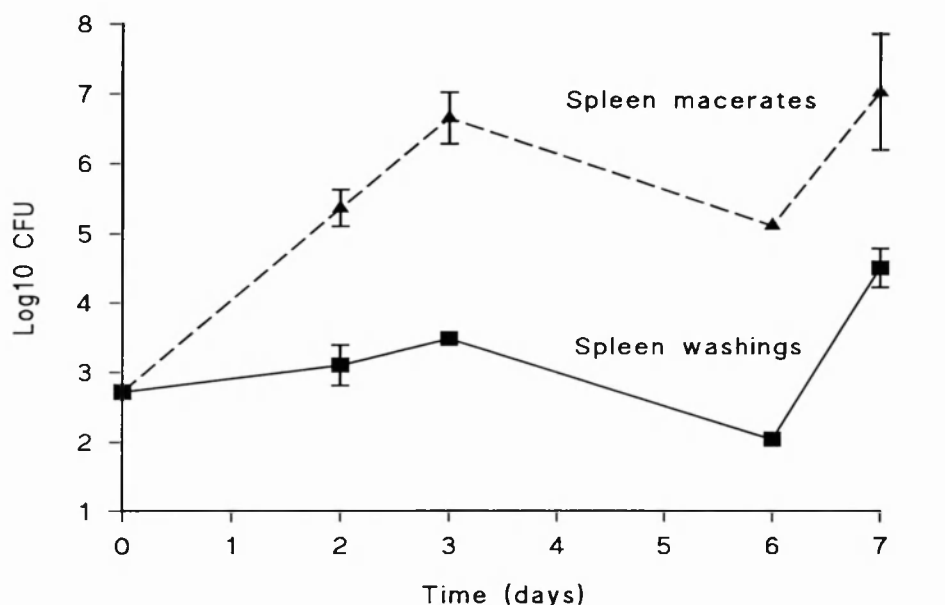


Figure 5.2 Comparison of *S. enteritidis* levels in spleen washings and macerates from moribund animals challenged with pH 4.35 cells

Table 5.7 A comparison of the proportion of *S. enteritidis* cells expressing flagella in the pH 4.35 challenge inoculum and in the population retrieved from spleen tissue

Population	Proportion Motile %	Expressing flagella %
pH 4.35 challenge	1	2
pH 4.35 retrieved	10 - 50	17

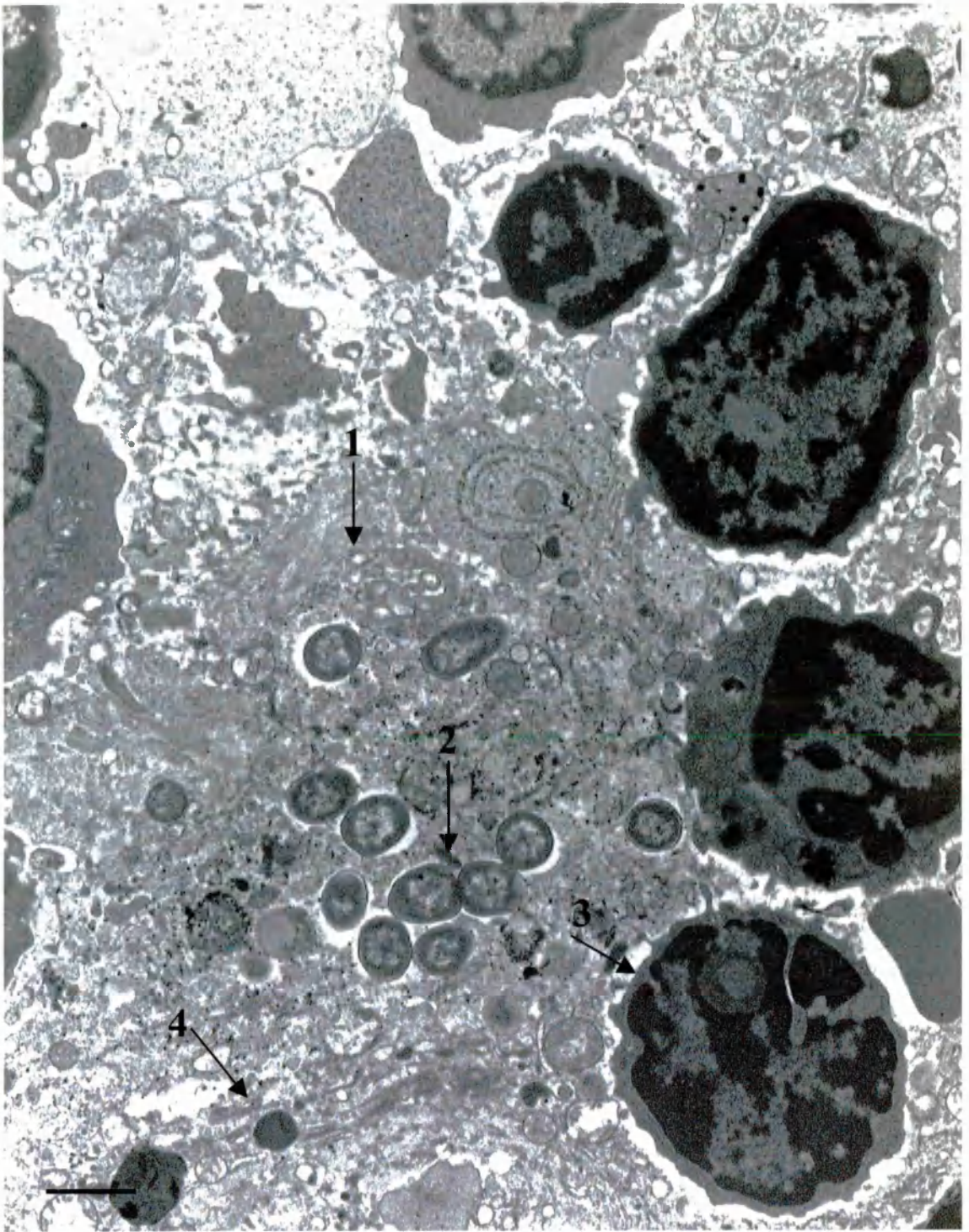


Figure 5.3 Transmission electron micrograph of spleen tissue infected with *S. enteritidis* PT4 grown at pH 4.35. 1 - host cell debris. 2 - intact, dividing *S. enteritidis* cells. 3 - lymphocyte - no *S. enteritidis* cells inside. 4 - lysosome. Bar = 1 μ m.

EM kindly prepared by AB Dowsett

5.4 Discussion

Compromises had to be made in the selection of a model for assessing virulence; when considering clinical disease the most relevant model is the human, but this was ethically unacceptable. The mouse model of salmonellosis has limitations; mice do not mimic the symptoms of disease displayed in humans, and in particular, gastroenteritis is not a feature of the murine disease. Mice are able to carry large numbers of bacteria within their tissues without any disease symptoms and do not usually die as a consequence of natural infection. In fact, in the natural situation, mice can act as a reservoir for *S. enteritidis* and can even be used as sentinel animals, indicating the level of contamination within hen houses etc. (Henzler and Opitz 1992).

Interactions with the commensal flora are important during the initial colonisation of a host. The normal microbiota of the gastrointestinal tract varies between animal species. The human microflora is essentially confined to the distal small bowel and large bowel, whereas mice harbour large microbial populations in the forestomach and throughout the intestinal tract. The flow dynamics through the gastrointestinal tract are also markedly different. Rodents eat in an almost uninterrupted fashion making the flow of contents more continuous than in the human caecum (Mackie *et al.* 1997).

Since infection with *S. enteritidis* is primarily by ingestion of bacteria, the oral route of inoculation was selected for its clinical relevance. However, the number of animals developing disease following oral inoculation was very low (Table 5.1). Other researchers have shown that strains of salmonellae which were virulent following intravenous inoculation were unable to initiate disease by the oral route in mice (Barrow and Lovell 1989). To use oral challenge, a concentration process would be required to obtain sufficient bacterial numbers from cultures grown at the pH limits (Table 3.1). This would involve a delay in the preparation of the inoculum causing the organisms to encounter different conditions. Environmental modulation could occur, changing the phenotype from that imposed by the defined growth conditions of the chemostat.

The intra-peritoneal (i.p) route of inoculation has disadvantages as it excludes some of the important stages of infection i.e. passage through the gastric barrier, adherence to the mucosal surface and translocation of the gut (Lax *et al.* 1995). However, i.p. infection of mice was more sensitive than oral dosing (Tables 5.1 and 5.2), providing a reproducible model for comparing the virulence of different populations of *S. enteritidis*.

A dose response was not always observed over the range of cell numbers tested (Tables 5.1 - 5.3). A non-linear response to infective dose has also been reported for *S. typhimurium*; the disease course in mice was shown to be slower following infection with intermediate cell numbers when compared to those infected with either high or low cell numbers. The results also indicated that a minimum number of bacteria are required before the host defences are activated (Briles *et al.* 1993). Data from outbreaks of human salmonellosis show the infective dose to vary over a wide range (Blaser and Newman 1982); and indicates some dose response, with larger inocula reducing the incubation period and leading to more severe symptoms (Mintz *et al.* 1994).

A challenge dose of approximately 10^6 cfu was selected, and this caused mortality proportions between 50% - 90% (Table 5.3). Also, no dilution was required to achieve this dose with cultures growing at the limits of pH which had cell densities of approximately 10^7 cfu ml⁻¹ (Table 3.1). Consequently, the bacteria were administered to the mice under the same environmental conditions as persisted in the chemostat and were not subjected to environmental 'shock' during a dilution stage.

Strains of *S. enteritidis* PT4 have been shown to exhibit marked differences in virulence for mice. Comparisons of the results are confused by the different methods of expressing virulence: LD₅₀, % survival and % mortality values are all routinely used. Some examples of the levels of virulence for *S. enteritidis* PT4 strains using mice are shown in Table 5.8.

Table 5.8 Determination of the virulence of *S. enteritidis* PT4 using mice

Route	Dose (cfu/mouse)	Virulence Determination	Reference
i.p	10 - 10 ⁷	LD ₅₀ < 12 cfu (LA5) LD ₅₀ ~ 10 ⁵ cfu (Se267) LD ₅₀ ~ 10 ⁷ cfu (<i>aroA</i> mutant)	(Cooper <i>et al.</i> 1992)
i.v.	range	LD ₅₀ = Log ₁₀ 1.3- 5.5	(Halavatkar and Barrow 1993)
Oral	2x10 ¹⁰	32% survival rate	(Peralta <i>et al.</i> 1994)
i.v.	10 ⁶	LD ₅₀ <10 cfu	(Cooper <i>et al.</i> 1990)
i.p.	range	LD ₅₀ < 20 (plasmid +ve) LD ₅₀ > 10 ⁶ (plasmid -ve)	(Chart <i>et al.</i> 1989)
Oral	10 ⁷	82% mortality (E) 10% mortality (I)	(Humphrey <i>et al.</i> 1996)
Oral	10 ⁷ 10 ⁶	0% mortality 10% mortality	this study
i.p.	4x10 ⁶ 6x10 ⁶ 3x10 ⁶	98% mortality (4.35)* 90% mortality (7.10)* 90% mortality (9.45)*	this study
i.p. = intra peritoneal i.v. = intra venous		* = growth pH Oral = gastric intubation	

When compared to published data for the virulence of other wild-type and mutant strains of *S. enteritidis* PT4 (**Table 5.8**), strain 226 405 was amongst those of lower virulence. This is not just a consequence of growth conditions as both batch and continuous cultures gave similar mortalities (**Tables 5.2 and 5.3**). Strain 226 405 is a clinical isolate, yet has low virulence in the mouse model. This may indicate that *S. enteritidis* has a greater infectivity for humans than for mice, emphasising that care must be taken when extrapolating from the mouse model to human disease.

The mouse model was able to discriminate between the virulence of *S. enteritidis* PT4 grown at different pH levels. Cells grown continuously at pH 4.35 were the most virulent both in terms of the total number of deaths and the rate of infection. The number of mortalities was significantly higher following challenge with pH 4.35 cells than with pH 9.45 cultures at day 14 ($p=0.04$). However, the difference between the virulence of the cultures was greater earlier, at days 3 and 4 post challenge the number of mice mortalities following dosing with the different pH cultures were all statistically significantly different, with pH 4.35 cells being the most virulent and pH 7.10 cells inducing the least number of deaths (**Table 5.4 and Fig. 5.1**). These data support the hypothesis made in **Chapter 4**, that the bacterial responses to high and low pH environments must differ.

The enhanced virulence and acid tolerance (**Tables 5.4 and 4.3, and Fig. 5.1**) of cells grown at pH 4.35 supports observations made by others. Isolates of *S. enteritidis* PT4 with enhanced tolerance of acid were more virulent in mice (Humphrey *et al.* 1996), whilst acid sensitive mutants of *S. typhimurium* demonstrated some degree of virulence attenuation in the mouse typhoid model (Foster and Hall 1990). An effective acid tolerance response has been strongly correlated with virulence (Riesenber-Wilmes *et al.* 1996). Induction of an acid tolerance response may enable the pH 4.35 cells to survive and replicate better in phagolysosomes thus enhancing and increasing the rate of invasion of the mouse. The presence of many virulence genes in non-pathogenic species suggests that the functions of these gene products is associated with survival in potentially lethal environments, rather than specific interactions with the host.

Enlarged spleens were observed in sick animals, with the mass of some organs having increased by approximately 3 fold (**Table 5.6**). The increased mass may indicate raised cellular immunological activity and be a consequence of macrophages assembling in an attempt to control the infection by ingestion of the invading *S. enteritidis* cells. Removal of circulating bacteria by the reticuloendothelial cells causes localisation of bacteria within the liver and spleen; these organs have been shown to be sites of *S. enteritidis* PT4 replication in mice (Halavatkar and Barrow 1993; Humphrey *et al.* 1997) and in infected chickens (Hinton *et al.* 1990; Cooper *et al.* 1994; Humphrey *et al.* 1996).

The level of intracellular bacteria within the spleen was higher than that contaminating the organ surface, confirming that the strain was invasive (**Figure 5.2**). Survival in the tissues implies resistance to the antibacterial effects of serum and this was demonstrated earlier for strain 226 405 (**Table 2.4**). Similar counts of bacteria were isolated from spleens of moribund animals irrespective of the growth pH of the inoculum. The expression of disease symptoms correlated with high levels of tissue invasion ($\sim 10^7$ cfu g⁻¹ of spleen). As expected the levels of bacterial carriage were lower in animals which did not display any symptoms at the end of the 14 days, i.e. approximately 10^4 cfu g⁻¹ of spleen (**Table 5.5**). This confirmed that disease symptoms were associated with high levels of tissue invasion. Infection may have progressed in these animals if the time period of the experiment had been extended.

Electron microscopy of spleen tissue from moribund, sacrificed animals suggested that cells of *S. enteritidis* PT4 were not present inside lymphocytes. However, some lysosomes were present, and there was also evidence of *S. enteritidis* PT4 cells being digested by phagosomes indicating a cellular response. Intact, healthy dividing *S. enteritidis* PT4 cells were observed but it was not possible to determine if these were within host cells and, if so, the type of cell (**Figure 5.3**). Host cell debris was seen around some *S. enteritidis* PT4 cells, possibly from lysed macrophages (**Figure 5.3**), suggesting intracellular division.

Following removal from the blood by macrophages within the spleen and other organs, bacteria are exposed to phagocytosis. If the bacteria are cleared from the blood their persistence within the macrophages determines the future course of the disease. Killing of the bacteria can terminate the infection, whilst bacterial growth can cause lysis of the cell, resulting in contamination of the neighbouring tissues and reinfection of the blood. Invasion and activation of macrophages by *S. enteritidis* has been demonstrated (Sasahara *et al.* 1992; Mizel *et al.* 1995). Survival and replication of *S. typhimurium* within macrophages is also well documented (Buchmeier and Heffron 1989).

Some interesting observations were made of the phenotypic characteristics of *S. enteritidis* PT4 *in vivo*. When pH 4.35 cells were inoculated into mice the proportion of flagellate cells increased (Tables 3.2 and 5.7). This may be an indication that flagella production was restored in response to *in vivo* stimuli and that flagella and or motility have a role in systemic infection of mice by *S. enteritidis* PT4. It has been demonstrated that *S. enteritidis* PT4 growing in the peritoneal cavity of chickens does not express flagella or SEF 14 (Chart *et al.* 1993). This may imply that the responses of *S. enteritidis* PT4 to the *in vivo* conditions of chickens and mice are distinct or that the signals which induced flagella production were experienced beyond the peritoneal cavity.

Most natural isolates of *Salmonella* have retained motility in face of the large energy demand imposed to maintain this function (Section 3.1.1). Only 1% of *S. typhimurium* isolates from a variety of human and animal sources were aflagellate (Duguid *et al.* 1975) again indicating a potential role for flagella and or motility in the natural life of the bacteria.

The role of flagella in the virulence of *S. enteritidis* is still under investigation. To date there have been conflicting reports on the role of flagella in the virulence of *S. typhimurium*; some researchers have reported a requirement for flagella for full virulence in mice following oral, i.v. or i.p. infection (Harshey and Toguchi 1996), whilst others have found no differences between motile and non-motile *S. typhimurium* cells in their ability to cause infection (Lockman and Curtiss III

1990). However, virulence attenuation was observed when mice were challenged via the oral route with mutants lacking both flagella and type 1 fimbriae (Lockman and Curtiss III 1992).

In contrast to continuous cultures, a high proportion of batch grown cells expressed flagella and type 1 fimbriae (Table 3.3), yet enhanced virulence was not demonstrated by these cultures (Tables 5.2 - 5.4). Two explanations can be put forward for this; either these surface structures do not have a major role in systemic infection, or as hypothesised above, the production of these surface structures is environmentally responsive and modulation of expression can be rapidly achieved in response to environmental signals. Due to the contaminating host material it was not possible to determine the proportion of the bacterial cells within spleen tissue which were expressing type 1 fimbriae by EM.

Fimbrial function in pathogenesis remains unclear, (Sections 3.1.2 and 3.4) and further investigations are required to determine whether changes in the levels of fimbrial expression occurred in response to specific host environments. It is difficult to speculate on the role of fimbriae from this study as only type 1 (SEF 21) fimbriae were identified and the i.p. route of infection was used. However, the data suggest that SEF 21 is not essential for infectivity of *S. enteritidis* PT4 in the murine model following i.p. infection, as the pH 4.35 cultures, which were most virulent, had only low proportions of bacteria expressing this fimbrial type (~20%, Table 3.3), and the proportion of cells expressing SEF 21 was higher in pH 7.10 cultures (~52% see Table 3.3), which were of lower virulence.

Similar observations have been made with *S. typhimurium* by comparing wild type cells and mutants which were unable to synthesise type 1 (SEF 21) fimbriae. When the virulence of wild type and mutant cells was compared using i.p. inoculation into a murine model no differences were observed. However, when oral challenge was used the nonfimbriated mutants produced significantly higher mortality, suggesting that SEF 21 fimbriae are not a virulence factor in *S. typhimurium* (Lockman and Curtiss III 1992).

Cells grown at pH 4.35 were the most virulent in the mouse model and caused high levels of tissue invasion (**Tables 5.4 and 5.5**), even though the level of carriage of the 38MDa plasmid by this population was lower than in cells grown at pH 7, and below the minimum detection level of the gel technique (**Fig. 3.5**). This observation conflicts with those indicating that the 38MDa plasmid is a prerequisite for full virulence of *S. enteritidis* PT4 in mice (Helmuth *et al.* 1985; Nakamura *et al.* 1985; Chart *et al.* 1989; Halavatkar and Barrow 1993).

Variations in the expression of the plasmid occur between strains. A naturally occurring plasmid negative strain and two plasmid-cured strains had higher LD₅₀ values when compared to plasmid carrying strains. However, introduction of the plasmid into the naturally plasmid-free strain did not subsequently increase the virulence of this strain when assessed using a mouse model (Halavatkar and Barrow 1993).

The clinical isolate of *S. enteritidis* PT4 used in this study demonstrated low virulence in the mouse model, when compared to other *S. enteritidis* PT4 strains (**Table 5.8**). The i.p. infective dose was high, and disease was generally not observed in mice following oral challenge (up to day 14) (**Table 5.1**). This may indicate that the virulence of this strain is attenuated by some other factor e.g. an impaired function of the RpoS transcriptional factor, which inhibits expression of the plasmid virulence genes (Guiney *et al.* 1995). This would also account for the introduction of the plasmid into a naturally plasmid negative strain not increasing the virulence i.e. if it was unable to express the plasmid genes.

Alternatively, the plasmid may have persisted in a low proportion of the population and subsequently been selected for when the relevant signals were received from the mouse environments. Therefore, the level of plasmid carriage in cells re-isolated from sick animals must be determined to confirm that in this investigation the 38MDa plasmid was having no significant role in pathogenicity.

It is becoming accepted that the plasmid genes are important for growth and colonisation of the spleen, liver and mesenteric lymph nodes following oral

inoculation of mice, and lead to a reduced lethal dose for mice (Gulig and Doyle 1993; Helmuth *et al.* 1993; Lax *et al.* 1993). Despite the increasing amount of data being accumulated on the molecular properties of the plasmids of salmonellae their potential role in salmonellosis remains an enigma. A large number of virulence genes are required for the intracellular survival of *S. enteritidis* in a host, the majority of which are distributed around the chromosome in pathogenicity islands (large clusters of virulence genes), although a small number do reside on the plasmid (Groisman and Ochman 1997).

The findings from murine models differ from those involving chickens where carriage of the plasmid had no significant effect on:- mortality, colonisation of the caecum, tissue invasion or translocation into eggs (Hinton *et al.* 1990; Halavatkar and Barrow 1993).

The plasmid has been associated with several phenomena related to virulence, including serum resistance and survival in macrophages. Again varying results have been reported and these may be due to strain variation; some researchers observed no effect of plasmid possession on the ability of *S. enteritidis* cells to grow in serum (Hovi *et al.* 1988; Suzuki *et al.* 1992); whereas others demonstrated that the presence of plasmids in *S. enteritidis* PT4 strains considerably reduced their ability to grow in the presence of chicken, turkey and human sera and they concluded that possession of plasmids may even be disadvantageous to certain bacteria in serum (Chart *et al.* 1996).

The role of the 38 MDa plasmid has not been associated with the acquisition of iron (Suzuki *et al.* 1992) or the expression of outer membrane proteins, flagella or fimbriae in the peritoneal cavity of the mouse (Chart *et al.* 1993).

The prevalence of virulence plasmids within strains isolated from infected organs was virtually 100%. In contrast, significantly lower frequencies occurred in strains isolated from the faeces of patients, asymptomatic excretors, food or the

environment (Helmuth *et al.* 1993; Nair *et al.* 1995). This confirms that expression of the plasmid genes is not a prerequisite for human disease.

In conclusion, this study has shown that the responsiveness of *S. enteritidis* PT4 to environmental pH makes a major contribution to the pathogenesis of the organism. Further investigations into the proteins which are under the control of environmental pH will be carried out in the following chapter.

Chapter 6

Changes in the Proteome and Phospholipid Content of *S. enteritidis* PT4 in Response to Growth pH.

6.1 Introduction

Enteric pathogens such as *S. enteritidis* PT4 exist in diverse natural habitats, (Section 1.11 and Figure 1.3), the conditions in which can be subject to rapid fluctuations. In general, bacteria have an exceptional capacity to survive and grow in hostile environments. Some groups of bacteria have even adapted specifically to life in extreme conditions e.g. thermophiles, halophiles and acidophiles. Although *S. enteritidis* is a neutrophile, growth can be sustained over a surprisingly wide pH range (Chapters 2 and 4), and growth pH has been shown to have major implications on the physiology (Chapter 3), survival (Chapter 4) and virulence (Chapter 5) of this organism.

Bacteria have relatively small genomes and do not possess tissues with specialised functions to facilitate survival. Consequently, to endure external flux an elaborate network of environmental sensors linked to response regulators has evolved. Proteins have a major role within these systems and enable bacteria to respond to environmental stimuli with maximum efficiency and minimum energy expenditure.

6.1.1 pH and protein activity

The proteome has been described as the total set of proteins being expressed by a genome (Wilkins *et al.* 1996); it has both structural and catalytic functions. Proteins also contribute to the general pH buffering capacity of the cell by virtue of their high content of weakly acidic and basic groups, attributable to the component amino acids.

Biological activity is generally attributed to one conformation of a protein. pH fluxes can induce conformational changes in proteins by affecting the electrostatic or hydrogen bonds which contribute to their three-dimensional structure. The structural changes can be minor or dramatic and may or may not alter the active sites of the protein. Some proteins can maintain their structure even at pH extremes and can also demonstrate optimum biological function at these pH levels. Small pH changes may not induce major structural changes, but can have significant effects on the activity of the protein, by altering the ionisation of a single dissociable group at the active site. The features of a protein structure which determine the specificity of regulation interactions are probably quite subtle (Stock *et al.* 1989).

6.1.2 Environmental awareness

Various environmental stimuli are received by bacteria including:- nutrient availability, the presence of other bacteria (quorum sensing, **Section 1.16**), host factors (**Section 5.1.1**), and parameters such as pH and temperature (**Sections 2.1.5.1 and 2.1.5.2**). Generally, environmental conditions are sensed within the cell by the effect of the agent on metabolism or cellular components.

'Two-component', transmembrane signal transduction systems are frequently used by bacteria for sensing environmental stimuli. These systems include pairs of proteins, namely, a sensor and a response regulator (Miller, J.F. *et al.* 1989; Parkinson and Kofoed 1992; Ninfa 1996). Most of the sensors are transmembrane proteins which continuously monitor specific environmental parameters, whereas the response regulators are cytoplasmic proteins (Parkinson and Kofoed 1992). Phosphorylation reactions between the proteins are used to regulate gene transcription, with the phosphorylated regulator being able to bind to DNA (Stock *et al.* 1989; Ninfa 1996).

6.1.3 Detection of environmental pH

The signal which initiates the sensing of external pH by bacteria is still unclear. There are many ways external pH can be detected by cells including changes in:- the structure and activity of cellular components, the ionisation state of specific

molecules, parameters such as the transmembrane pH difference (Δ pH), proton motive force (pmf) and the membrane potential (ψ).

A diagrammatic summary of the potential routes for sensing external pH by bacteria is shown in **Figure 6.0**. The situation is probably more complex, with the existence of additional components which interconnect into other regulatory circuits and feedback loops.

Environment

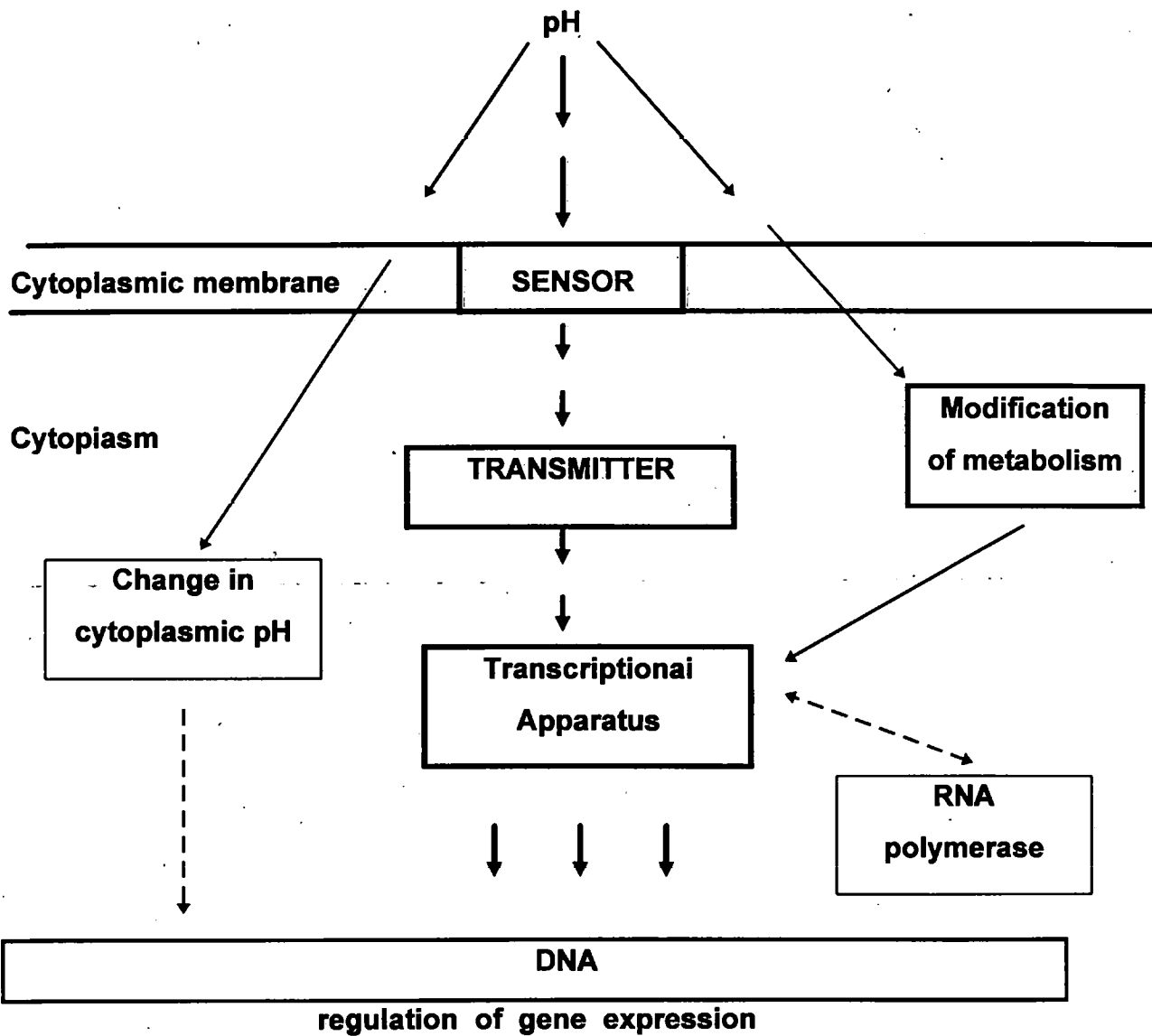


Figure 6.0 A schematic representation of the detection and regulatory responses to environmental pH

It has been postulated that phosphate and the proteins involved in its transport and metabolism are implicated in the sensing of pH flux by *E. coli* (Rowbury *et al.* 1992). This hypothesis is supported by the observation that 11 of the 52 proteins induced in *S. typhimurium* by phosphate starvation were also induced following acid shock (Olson 1993).

6.1.4 Responses to external pH

Bacteria respond to changes in external pH by adjusting the activity and synthesis of proteins associated with many different processes. Bacteria elicit specific and rapid changes in metabolic activity and gene expression in response to a range of environmental stresses including acidic or alkaline pH. ΔpH is positive under acid conditions and becomes negative when cells encounter high pH. ΔpH is a component of the proton potential of the cell which drives the processes of transport, motility and the coupling of respiration. Therefore, changes in the nature of the ΔpH value influences all of these processes.

The effects of environmental pH on cell functions fall largely into two categories:- (1) physiological responses, such as chemotaxis where changes in external pH are detected, but do not result in alterations in the expression of specific genes *per se*, and (2) genotypic responses, where regulation of gene expression is a direct consequence of the external pH.

6.1.5 Regulation of internal pH

For the majority of bacteria intracellular pH homeostasis is essential for the maintenance of bacterial metabolism including enzyme functions (Booth 1985). Variations in the external pH can stimulate metabolic changes which act to maintain intracellular pH homeostasis e.g. amino acid deaminases and decarboxylases are induced by high and low pH, respectively (Gale and Epps 1942; Watson *et al.* 1992). Amino acid decarboxylases remove the acid groups reducing the acidification from metabolic processes (Gale and Epps 1942; Hall *et al.* 1995).

Control of cytoplasmic pH can also be achieved by regulation of the activity of ion transport systems which facilitate proton entry or extrusion. At alkaline pH, Na^+/H^+ antiporters are switched on to lower the internal pH (Pinner *et al.* 1992; Olson 1993). In order to maintain the internal pH at a functional level over a wide range of external pH values a sensor system coupled to a mechanism for regulation of gene expression is required.

6.1.6 Motility and chemotaxis responses

The direction of flagellar rotation is controlled by the proteins of the flagellar motor which is situated at the base of the structure. Cell motility and chemotactic responses are determined by signals initiated by environmental stimuli which are detected by the signal transduction proteins. Subsequent modulation of the proteins in the flagellar motor can be used to re-orientate the direction of swimming, enabling bacteria to migrate away from hostile conditions such as extreme pH levels (Kihara and Macnab 1981; Macnab 1996).

6.1.7 pH regulation of gene expression

pH regulated gene expression has been studied in enteric bacteria, and since 1980 many genes which are regulated by external pH have been identified. Evidence is available for the involvement of:- external pH (pH_o), internal pH (pH_i), ΔpH ($\text{pH}_o - \text{pH}_i$), ψ and pmf in altering gene expression (Hall *et al.* 1995).

The complex nature of bacterial processes requires a large number of genes to be under both independent regulation and co-ordinated control. Operons enable bacteria to co-regulate genes which have related functions e.g. those coding for proteins involved in the same metabolic pathway. The number of genes which can be accommodated in an operon is limited. Therefore, regulation at levels beyond the operon, i.e. at the regulon level, is required to enable bacteria to grow and survive under many conditions. The molecular responses to environmental conditions at the whole-cell level have been termed global control (Gottesman 1984).

All the genes which respond to an environmental stimulus may not be regulated by the same protein, but may belong to a number of regulons which respond

independently to the same signal. Such a group of regulons is known as a stimulon (Neidhardt 1987). Some operons belong to more than one stimulon which accounts for the interdependency of some of the stress responses observed in **Chapter 4** (Neidhardt and Van Bogelen 1987). Most responses of bacteria to external stimuli involve multiple regulons and the production of repressors and activators that control independent operons (Neidhardt 1987; Neidhardt and Savageau 1996).

Regulation of gene expression can be a consequence of changes in mRNA stability and translation, and in protein stability, folding, secretion and activity (Stock *et al.* 1989; Ninfa 1996). Bacteria are constantly regulating the information transfer from the genome to the ribosomes. The half life of mRNA molecules is only a few minutes, thus enabling new information to continuously enter the gene regulation system. A change in the internal pH may also directly influence the conformation of the DNA and alter regulatory sites (Neidhardt and Savageau 1996).

6.1.8 The SOS response

Exposure to certain hostile conditions which cause damage to DNA results in the induction of more than twenty proteins in a diverse set of physiological responses which is termed the SOS response (Dorman 1994). The SOS response of *E. coli* has been shown to be induced as a result of increased intracellular pH (Schulinder *et al.* 1986). However, the acid tolerance response (ATR) of *S. typhimurium* appeared to be specific to acid stress giving no cross-protection for SOS function (Foster and Hall 1990).

6.1.9 Stress proteins

Stress proteins have been observed in all living organisms. They are one of the protection strategies used to prevent cell death from the harmful effects of extreme environmental factors. When activated, these proteins may act rapidly to protect vital biochemical functions inside the microbe. Frequently, stress proteins are expressed under optimal conditions, and are up-regulated in response to a stress stimulus.

Induction of acid tolerance requires protein synthesis and involves changes in the levels of a number of proteins (Foster 1993; Humphrey *et al.* 1993a; Wu *et al.* 1994; Bearson *et al.* 1996; Rowbury *et al.* 1996). These specialised adaptation and survival mechanisms involve the expression of stress proteins which have functions such as:- molecular chaperones (Langer *et al.* 1992), (**Section 6.1.10**); protection of cellular proteins from denaturation (Ericsson *et al.* 1994); and regulation of gene expression (Stock *et al.* 1989) (**Section 6.1.7**).

6.1.10 Molecular chaperone proteins

The biological function of newly formed polypeptide chains is controlled by the three dimensional structures into which they fold (**Section 6.1.1**). In bacterial cells, proteins are built by the formation of polypeptide chains which are elongated as translation proceeds. The growing chains are susceptible to misfolding and aggregation. A number of proteins, termed molecular chaperones, have been identified which are able to bind to the unfolded, nascent polypeptide chains and catalyse their correct folding (Langer *et al.* 1992). The chaperone proteins are non-specific, and bind to a wide range of unfolded or partially folded proteins. The attachment of the chaperones is transient, as they do not form part of the final protein. The polypeptide chain is released in a controlled manner so that the required folding is achieved (Bochkareva *et al.* 1988; Langer *et al.* 1992).

Although chaperone proteins have essential “house-keeping” functions (including protein translocation), their expression is also inducible by a range of stresses, with a number of them being heat shock proteins, including DnaK, DnaJ and GroEL, which are members of the heat shock protein (hsp) families 70, 40 and 60, respectively (Langer *et al.* 1992).

6.1.11 Extracellular proteins

Bacteria can also interact with their environment by the excretion of proteins such as:- proteases, which are responsible for the breakdown of macromolecular substrates into transportable size units; invasins, to facilitate cellular penetration; toxins, which are frequently active against host cells, and bacteriocins to inhibit bacterial competitors. The external pH will influence the activity of these extracellular proteins and, consequently, their role in bacterial processes.

6.1.12 Outer membrane components

The outer membrane forms a barrier between the external and internal environments of the bacterium. This is not a static role, but is a dynamic function which supports a range of cell processes which are essential to the survival and pathogenicity of the cell. The outer membrane of *S. enteritidis* contains two types of lipids, namely lipopolysaccharide and phospholipids, as well as a set of characteristic proteins. The phospholipid composition of the outer membrane is similar to that of the cytoplasmic membrane, with a slight enrichment of phosphatidylethanolamine (Osborn *et al.* 1972). The composition of phospholipid varies with bacterial species and growth conditions.

6.1.13 Phospholipids

Phospholipids have multiple roles in bacterial cells, with their primary function being to establish permeability barriers around the cell. A large number of proteins are associated with the phospholipid layers and these include, proteins involved in environmental sensing, transport, energy transduction and cell-cell recognition (Dowhan 1997). The physical state of the membrane lipids influences the properties of the embedded proteins, with many membrane enzymes being stimulated by the presence of particular phospholipids (Müller, E. *et al.* 1972). Consequently, alterations of the phospholipid composition of the membrane is important in membrane function.

6.1.14 Aims

The aim of this study was to determine the influence of growth pH on the proteome and phospholipid content of *S. enteritidis* PT4. Proteins perform major roles in bacterial physiology, survival and virulence, all of which were shown to be influenced in *S. enteritidis* PT4 by growth pH (Chapters 3, 4 and 5). Consequently, identification of proteins which are regulated in response to environmental pH may highlight correlations between protein functions and survival strategies, and/or virulence determinants. The phospholipid composition of the cells will also influence the properties of a vast array of proteins and will

consequently induce changes in key cell processes such as transport and environmental sensing (**Section 6.1.13**).

The specific aims of the investigation are to:-

1. Use 2D gel electrophoresis techniques to obtain two dimensional reference maps of the protein profile of cells grown under controlled conditions in a chemostat at the pH limits for growth, i.e. pH 4.35 and pH 9.45, and at pH 7.10 for comparative purposes, (as described previously in **Section 3.2.2**).
2. Compare the proteome maps of cells grown at different pH levels and target protein spots which are regulated in response to growth pH for N-terminal amino acid sequencing.
3. Search software databases for homologous proteins with known identity and function.
4. Ascribe possible roles for the regulated proteins in the survival and/or virulence of *S. enteritidis* PT4.
5. Compare the phospholipid content of cells grown at different pH values.

6.2 Methods

6.2.1 Bacterial strain and growth conditions.

S. enteritidis PT4 strain 226 405 was grown continuously at pH 4.35, pH 7.10 and pH 9.45 in Vogel Bonner medium (Vogel and Bonner 1956), supplemented with 2.5g l^{-1} hog gastric mucin and vitamins using the techniques described in **Chapter 3** sections **3.2.1** and **3.2.2**. All other environmental parameters were maintained constant; the temperature was set at 37°C and the cultures were grown under an atmosphere of 5% CO_2 in nitrogen, and the medium flow rate was set to give a dilution rate of 0.1h^{-1} (which is equivalent to a mean generation time of 6.9h).

The effluent from the chemostat vessel was collected over 2 hours to obtain sufficient cell mass. The effluent was collected into a flask on ice containing 1ml of inhibitor solution which contained 1mgml⁻¹ of chloramphenicol (Sigma, UK) and 50mM phenylmethylsulphonyl fluoride (PMSF, Sigma, UK). Protease activity was inhibited by the PMSF and *de novo* protein synthesis was prevented by the action of chloramphenicol. Cell pellets were prepared from 100 ml samples of collected cultures by centrifugation at 3,000g for 10 min. Cell pellets were stored at -20°C until processed.

6.2.2 Cell lysis and solubilisation of the whole cell protein

Cells were washed by resuspending pellets in 0.5ml of sterile saline, and then centrifuged at 2,000 g in a microfuge prior to discarding the supernatant. 100µl of lysis buffer was added to pellets from the pH 4.35 and pH 9.45 chemostats, and 500µl to pellets from the pH 7.10 chemostat. The pellets were resuspended in the lysis buffer and left at room temperature until the gel system was ready for loading the samples. Lysis buffer was prepared as follows:-

Urea (Sigma, UK) - 13.5g, Triton X-100 (Sigma) - 0.5ml, dithiothreitol (Sigma) - 500mg, Pefabloc (Merck) - 5mg, Pharmalyte (Pharmacia) 3-10 - 0.5ml. The urea was dissolved in ~ 20ml of distilled water before adding the remainder of the constituents; finally, the volume was made up to 25ml with distilled water.

6.2.3 Two-dimensional gel electrophoresis

Two dimensional gel electrophoresis separates hundreds of polypeptides by their isoelectric points and molecular masses (O'Farrell 1975). The technique can be used to obtain two dimensional reference maps for proteins being expressed by cells from different environmental conditions. Comparison of the maps indicates which proteins are expressed *de novo*, which are up-regulated or which are suppressed.

This was performed using a Multiphor II (Pharmacia, Uppsala, Sweden) horizontal unit with immobilised pH gradient gels covering the range pH 3 - pH 10 (Pharmacia) in the first dimension. The wider pH range gels were selected to cover more pI values. The longer strips were also selected as they have a

greater separating power. Sodium dodecyl sulphate polyacrylamide gradient gels covering the range 8-18% polyacrylamide were used in the second dimension as these gave sharper spots. The electrophoresis was carried out as described in the Pharmacia instructions manual (Pharmacia 1995).

The optimal load per gel was estimated to be approximately 5×10^9 *S. enteritidis* cells when the gels were to be stained with Coomassie blue (Sigma), (see 6.2.5).

6.2.4 Developing

After electrophoresis in the second dimension, the gel was rocked gently in a developing solution of 40% v/v ethanol, 10% v/v acetic acid for 30 min.

6.2.5 Staining

The protein spots were then visualised by staining with Coomassie blue at 60°C for 10 min. Heating the stain reduced the time required for the staining to be completed. The background stain was removed by subsequently rocking the gel in destain (25% v/v ethanol, 8% v/v acetic acid).

6.2.6 Preserving

After destaining, the gels were placed into preserving solution for 30 min. This was 250ml of the destain solution plus 25ml of glycerol. The gel was then placed on a glass slide, gel side upwards, and covered in a cellophane preserving sheet soaked in preserving solution. When the sheet had dried onto the gel, the glass plate was removed and the edges around the gel were sealed with tape.

6.2.7 Standards

The commercially available gels (Pharmacia) are prepared to have a uniform pH gradient along their length, to make isoelectric focusing possible in the first dimension. The proteins are then separated by molecular weight when the gels are run in the second dimension. The molecular weight of a protein can be determined by comparing its electrophoretic mobility, (R_f) with those for known protein markers. An exponential relationship exists between molecular weight and (R_f).

$$R_f = \frac{\text{distance protein has migrated from the origin}}{\text{distance from the origin to the dye front}}$$

A calibration curve of MW against R_f values was made.

In order to confirm the accuracy of pI and MW determinations made using the 2D gel system described, a set of 2-D SDS-PAGE protein standards, (Bio-Rad), with known pI and MW values were run, and actual and estimated values compared. The standards covered the pI range pH 4.5 to pH 8.5 and the molecular weight (MW) range 17,500 to 76,000 daltons (Table 6.1).

Table 6.1 Standards used to assess the accuracy of molecular weights (MW) and isoelectric points (pI) determined from 2-D SDS-PAGE gels

Protein	MW (KDa)	pI (pH)
Hen egg white conalbumin type 1	76.0	6.0 6.3 6.6
Bovine serum albumin (BSA)	66.2	5.4 5.6
Bovine muscle actin	43.0	5.0 5.1
Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase	36.0	8.3-8.5
Bovine carbonic anhydrase	31.0	5.9 6.0
Soyabean trypsin inhibitor	21.5	4.5
Equine myoglobin	17.5	7.0

MW standards were included at both ends of each gel (Low molecular weight calibration kit for electrophoresis, Pharmacia, Table 6.2). The inclusion of

standards in each gel run provides an internal control for the reproducibility of 2-D experiments and compensates for any variation in the distance the front travels.

Table 6.2 Molecular weight standards included in second dimension runs

Protein	MW (KDa)
Phosphorylase b	94.0
Bovine serum albumin	67.0
Ovalbumin	43.0
Carbonic Anhydrase	30.0
Trypsin inhibitor	20.1
α-Lactalbumin	14.4

6.2.8 Comparison of proteome maps

Coomassie blue stained gels were used for comparing the protein profiles and for determining the MW and pI values of proteins by their electrophoretic position. The gel patterns revealed which proteins were being expressed (O'Farrell 1975), while the size and integrated intensity of the spots indicated the amount of protein present. Integrated intensities were compared as this compensates for any variation in the size of the initial sample loaded onto the gel. The Bioimage system was used to analyse the gels.

6.2.9 Protein spot blotting

When the gel had been run in both dimensions, it was either stained as described above, or was blotted to transfer the protein spots onto PVDF transfer membranes (PolyscreenTM, Biotechnology Systems, NEN Research Products, Boston MA), ready for N-terminal microsequencing. The blotting method was based on that described by Matsudaira (1987) and was as follows:

1. 4 sheets of blotting paper were soaked in electroblotting buffer (see below).
2. One sheet of Problott membrane was wet in methanol prior to transferring to electroblotting buffer.
3. After removing from the electrophoresis tank, the gel was soaked in electroblotting buffer for 5 min. The gel was then carefully removed from the plastic backing sheet. A corner was removed from the gel so that the orientation was known.
4. Two sheets of blotting paper were placed on the lower electrode of the blotting apparatus (Semi-dry electroblotter B, Ancos, Denmark). The sheets were gently rolled to exclude any air bubbles. The PVDF membrane was removed from the buffer and laid over the cut gel. The gel was kept as flat as possible.
5. The gel was then turned into the apparatus so that the membrane was on top of the blotting paper.
6. The remaining two strips of blotting paper were placed over the top of the gel. A tube was rolled very gently over the sandwich to remove any air bubbles.
7. The electrode assembly was completed and the electroblot system was run at 80mA for 1.5h.
8. After electroblotting, the membrane was washed in distilled water for 2 min. prior to staining.

Staining the membrane:

1. The membrane was soaked in methanol for a few seconds, and then rocked in 100ml of amido black stain (see below), for 1 min.
2. The membrane was rinsed with water, then placed in 100ml of de-stain (see below).
3. De-staining was repeated 3 times until good contrast was obtained between the stained protein spots and the white background.
4. The Problott membrane was then dried, and protein spots excised as required

Solutions:

Stock CAPS buffer:

22.13g CAPS were dissolved in 900ml of distilled water, the pH was adjusted to pH 11 with 2M NaOH, and the final volume was made up to 1 litre with distilled water.

Electroblotting buffer:

50ml of stock CAPS buffer plus 50ml of methanol plus 400ml of distilled water.

Amido black stain:

0.1% (w/v) Amido Black (Naphthol blue black, electrophoresis reagent, Sigma) was dissolved in 1% acetic acid / 40% methanol (v/v).

De-stain:

150ml ethanol plus 30ml of acetic acid plus 120ml of distilled water.

6.2.10 N-terminal microsequencing

Protein spots excised on the Problott membrane were placed in the aperture of a protein sequencer (Applied Biosystems, model 477A); the dimensions of the aperture restricts the number of membranes which can be inserted at one time.

The identified sequence was compared to known proteins using Swiss-Prot, Release 34 database which identifies the closest homologues. The software determines the number of amino acids which overlap and their position from the N-terminal end, and the probability of the identification (% identification).

6.2.11 Polar lipid extraction

Polar lipids were extracted by a modification of the procedure of Bligh and Dyer (1959). 10 ml samples of culture removed directly from the chemostat vessel were freeze-dried and used for extraction. The lyophilized biomass was suspended in 2 ml of chloroform/methanol/distilled water (1:2:0.8) and vortex mixed for 2 minutes. A further 0.6 ml of chloroform was added and the mixture was vortexed for 0.5 min. Separation of the phases was encouraged by low speed centrifugation, after which the lower (mainly chloroform) layer was removed with a Pasteur pipette to a clean tube, and washed with ultra high quality water. The water washes were discarded, and the residual organic layer was evaporated in a vacuum centrifuge and redissolved in 30 μ l chloroform/methanol (2:1), prior to analysis by fast atom bombardment mass spectrometry (See 6.2.12).

6.2.12 Fast atom bombardment (FAB) mass spectrometry

FAB mass spectra of polar lipid extracts were recorded in both positive and negative ion modes with a Kratos MS80RFA spectrometer (Manchester, UK) equipped with an Ion Tech FAB gun using xenon atoms as the bombarding particles. The liquid matrix used was 1 μ l 3-nitrobenzyl alcohol mixed with an equal volume of sample solution on the stainless steel target. The instrument was operated at 4kV accelerating voltage and scanned at 10 seconds per decade over the range 1800-100 under the control of Mach-3 software running on a Sun Microsystems SparcStation IPX. The instrument resolution was 1000 in all cases. The individual phospholipid species were identified by means of collision-induced dissociation (CID) and linked scanning experiments (in the positive ion mode only). Collision induced dissociation was performed using helium as the collision gas, at a pressure sufficient to attenuate the precursor ion beam to 50% of its initial intensity. The products of collision in the field free region between the source and the electrostatic analyzer were recorded by means of computer controlled linked scans under the control of the Mach 3 software. Profiles of the individual types of phospholipids in complex mixtures were obtained by means of constant neutral loss experiments in which the mass of the neutral fragments was characteristic of a given polar head group. Thus scans for the loss of the neutral species 141, 155, 169 and 172 were used to selectively record phosphatidylethanolamines, *N*-methyl phosphatidylethanolamines, di-*N*-methyl-phosphatidylethanolamines and phosphatidylglycerols, respectively. Phospholipids (such as phosphatidylcholine), in which charge is retained on the polar head-group fragment, were identified by B/E linked scans, using the protonated molecule as the precursor ion.

6.3 Results

6.3.1 Estimation of the accuracy of pI and MW values determined using 2D gel electrophoresis

Protein standards with known molecular weight and pI values (Table 6.2) were separated using the 2D-electrophoresis method as described earlier (Section 6.2.3). The actual and determined MW and pI values are shown in Table 6.3. These values indicate that the electrophoresis methodology does allow for

relatively accurate preliminary characterisation of proteins in terms of pI and MW values. Some variation in the MW determinations did occur, but the variation was usually less than 15%. Generally, pI estimations were in close correlation with the standards, although the pH gradient was less accurate across the range pH 6 to pH 7, with the estimated pI values in this region being higher than the actual values (Table 6.3). The deviations observed within the values determined for protein standards indicate that confirmation of gel linearity should be made each time by the inclusion of protein standards with test samples.

Table 6.3 A comparison of the actual and estimated pI and MW values using protein standards

Protein ref. No. (Table 6.1)	MW actual (KDa)	MW determined (KDa)	pI actual	pI determined
1	76.0	70.0	6.0-6.6	7.2
2	66.2	66.4	5.4-5.6	5.7 5.6
3	43.0	45.5	5.0-5.1	4.8
4	36.0	46.0	8.3-8.5	8.2
5	31.0	36.3	5.9-6.0	6.7
6	21.5	25.1	4.5	4.2
7	17.5	15.1	7.0	8.1

6.3.2 Determination of the variability in MW estimations between gels

The R_f values for proteins standards (Table 6.1) were calculated from five independent gels and a calibration curve prepared; an approximately linear relationship was obtained if log MW was plotted against R_f (Fig. 6.1). Generally, the reproducibility of the calibration curves from separate gels is very good. A linear regression of all the data was calculated and is shown in Fig. 6.1. The

values determined experimentally do not generally deviate far from the regression line, again emphasising the good reproducibility of MW estimations between gels. The equation for the regression relationship between MW and R_f value in this system was :-

$$\log MW = -1.86R_f + 5.78$$

regression coefficient $r^2 = 0.98$

This equation describes the calibration curve between protein molecular weight and electrophoretic mobility and can be applied to determine molecular weight values using experimentally determined migration values. The greatest deviations from the linear relationship occurred at the limits of the range of MW estimations. The arrows indicate the limits of the MW range over which a linear relationship exists (approximately 20-70 Kda) (Fig. 6.1). Again this deviation emphasises the requirement to include protein standards in each run, especially when considering proteins across the entire MW range of the gel. Application of the calibration equation will provide a good estimation of MW values which fall within the linear region.

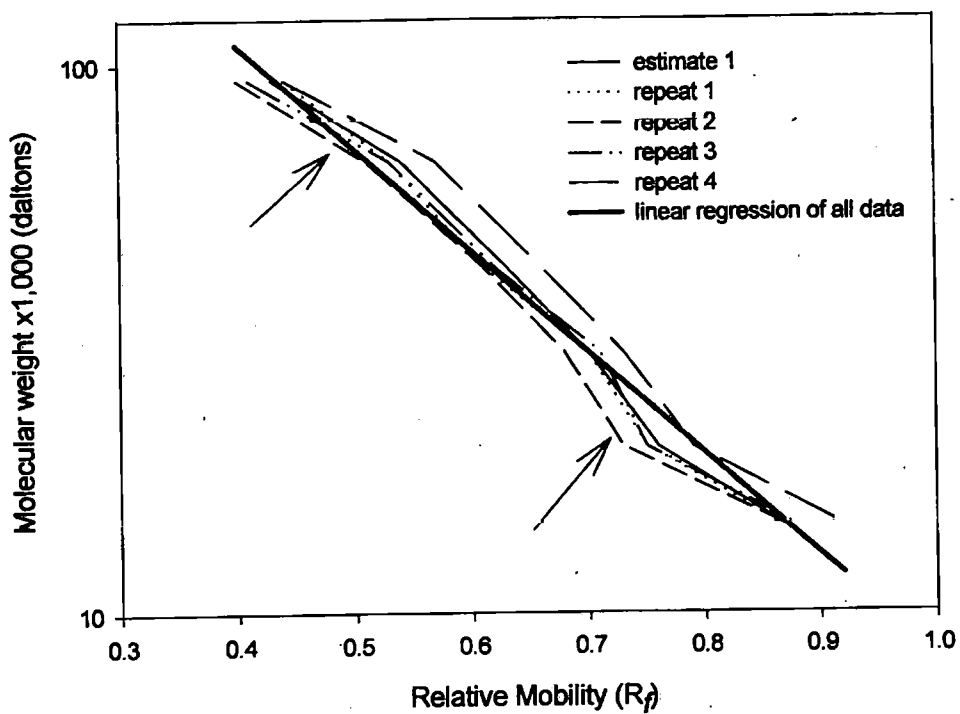


Figure 6.1 A calibration curve for R_f values against MW using protein standards on a pH 3 - pH 10 gradient gel.

6.3.3 Comparison of the proteome maps of cells grown at different pH levels

The proteome maps obtained using 2D gel electrophoresis and Coomassie blue staining for cells grown at pH 4.35, pH 7.10 and pH 9.45 are shown in **Figure 6.2**. Some of the proteins which are regulated in response to external pH are indicated.

Table 6.4 A comparison of the proteomes of cultures of *S. enteritidis* PT4 grown at different pH levels

Growth pH	Total No. of spots	No. of high intensity spots*	No. of large spots♦
4.35	~215	65	26
7.10	~200	80	33
9.45	~200	73	20

* integrated intensity >0.5%

♦ integrated intensity >0.8%

The total number of spots can only be estimated as the intensity and size of some of the spots is very small. The values varied when repeated scans of gels were performed. The value will also be dependent on the loading and staining efficiency. In this study, only the more intense spots have been targeted for further investigation.

The number of large and intense spots was highest for cells grown at pH 7.10. This indicates that the production of some proteins must be down-regulated in response to growth at extreme pH values. The number of proteins with altered expression levels between cells grown at different pH levels is shown in **Table 6.5**.

Table 6.5 The number of proteins with altered levels of expression at the limits of growth pH when compared to pH 7.10 cultures

Regulation of protein expression when compared to pH 7.10 cultures	Growth pH	
	pH 4.35	pH 9.45
number of regulated proteins	16 ± 2 (3)*	32 ± 8 (3)
Proportion of proteins up-regulated	58%	46%
Proportion of proteins down-regulated	42%	54%
Number of novel proteins	1	4

* number of proteins ± standard deviation (n = replicates)

Preliminary characterisation of some of the proteins which were regulated in response to external pH was performed and possible identities ascribed by comparing their electrophoretic properties with those of *S. typhimurium* (Table 6.6) (Qi *et al.* 1996). One of the proteins which was up-regulated in response to growth at pH 7 was possibly a precursor of PhoE which is an outer membrane pore protein which is inducible by phosphate-limitation (Spierings *et al.* 1992). PhoE has been implicated in acid habituation, with H⁺ or protonated carriers crossing the outer membrane via the PhoE pore (Rowbury *et al.* 1992). Two of the proteins which were up-regulated at pH 7 were associated with flagella assembly and function (Tang and Blair 1995), whilst another ATP synthase is an enzyme involved in energy metabolism (Table 6.6). The N-terminal sequences of these proteins are still to be determined to identify homologues.

Table 6.6 Preliminary characterisation of proteins which are regulated in *S. enteritidis* by external pH

Spot Number	pI	MW (KDa)	pH at which expression is increased	Possible identity
10	4.40	47.0	4, 9	-
14	5.10	94.7	4	-
15	4.07	18.3	4	-
21	4.84	51.6	7	subunit protein of phase 1 flagella
30	7.59	22.4	4	-
45	3.97	14.0	4	-
46	3.95	14.0	4	-
71	4.43	25.5	7	-
72	4.95	26.8	7	-
73	4.67	37.6	7	PhoE precursor of <i>S. typhimurium</i>
74	4.81	44.6	7	-
75	4.87	50.4	7	ATP synthase β subunit
76	5.14	38.5	7	FliM protein of <i>S. typhimurium</i>

6.3.4 Characterisation of selected proteins

A range of proteins have been targeted for possible identification using both electrophoretic properties and N-terminal micro-sequencing. These have been given references SES 1 - SES 7 and are labelled 1-7 in **Fig. 6.2**. The properties of these proteins, as determined from their electrophoretic positions and intensities, are shown in **Table 6.7**.

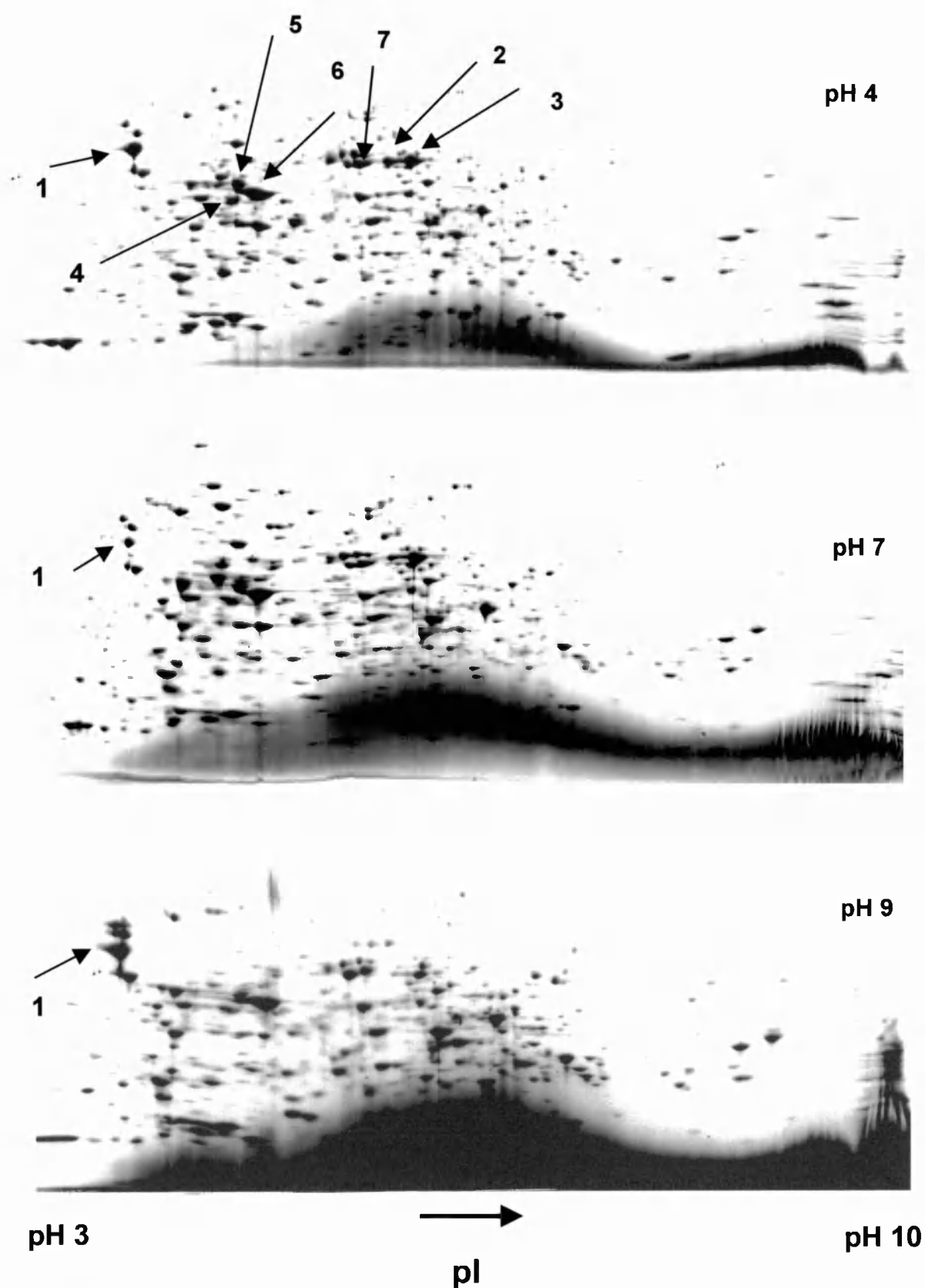


Figure 6.2 The proteome maps of *S. enteritidis* grown at pH 4.35, pH 7.10 or pH 9.45 on 2D gels stained with Coomassie blue

Table 6.7 The expression of targeted proteins in cells grown at different pH levels

Protein reference	MW (KDa)	pI	Relative intensity of protein expression by cells grown at:		
			pH 4.35	pH 7.10	pH 9.45
SES1	60.1 ± 2.7	4.3 ± 0.19	++	+	+++
SES 2	56.1 ± 2.9	6.0 ± 0.54	++	+	±
SES 3	50.4 ± 0.6	5.9 ± 0.18	++	++	+
SES 4	37.8 ± 0.2	5.1 ± 0.02	++	+	+
SES 5	39.4 ± 7.6	5.2 ± 0	++	+	+
SES 6	37.6 ± 4.4	5.3 ± 0.04	+++	+++	+++
SES 7	57.2 ± 3.3	5.9 ± 0.06	++	+	+

The molecular weight and pI values are means of three determinations
± standard deviation

The homologues identified from the N-terminal sequences of the proteins are shown in **Table 6.8**, and the sequence alignment data in **Table 6.9**.

Table 6.8 Polypeptides assigned to the selected proteins from the 2D maps

Protein spot reference	Sequence homologue	% identification	No. of amino acid overlaps
SES 1	GroEL from:		
	(<i>Actinobacillus actinomycetemcomitans</i>)	93.3	15
	(<i>E. coli</i>)	93.3	15
	(<i>S. typhimurium</i>)	93.3	15
SES 2	Unidentified due to equipment failure		
SES 3	Cystathionine gamma-synthase (<i>Haemophilus influenzae</i>)	62.5	8
	Pyrrolin-s-carboxylate reductase (<i>Corynebacterium glutamicum</i>)	71.4	7
	RecA protein (<i>Deinococcus radiodurans</i>)	85.7	7
SES 4	Leu/ileu/val binding protein precursor (<i>E. coli</i>)	91.7	12
SES 5	Enolase (<i>E. coli</i>)	93.3	15
	(<i>H. influenzae</i>)	93.3	15
	(<i>Zymomonas mobilis</i>)	46.7	15
SES 6	N-terminal blocked		
SES 7	Novel		

Table 6.9 Sequence alignment of the N-terminal amino acid sequences of the selected proteins with their homologues

Protein spot or homologue	Sequence alignment	Position of the residues from the N-terminal
SES 1 GroEL	AGKDV KFHNDARV KM AAKDV KFGNDARV KM	1
SES 2	No data	
SES 3 Cystathionine gamma -synthase	GQE <u>I</u> XDR <u>V</u> GQE <u>V</u> LDRI	218
SES 4 LEU/ILEU/VAL-binding protein precursor	EDIKVAVVGAT <u>SS</u> EDIKVAVVGAM <u>SG</u>	24
SES 5 <i>E. coli</i> enolase	SKIVK <u>V</u> IGREI DSR SKIVK <u>I</u> IGREI DSR	1
SES 6	N-terminus blocked no sequence determined	
SES 7	KQLVYLSE Novel	

Identical amino acid matches are shown in bold and conservative amino acid replacements are underlined.

Homologous proteins were identified to only three of the targeted proteins, SES 1, SES 4, and SES 5 (Tables 6.8 and 6.9). Although a homologue was identified to protein SES 3, the closeness of the match was too low to be acceptable. SES 7 was a novel protein with no sequence match in the Swissprot database.

6.3.5 Comparison of the phospholipid content of *S. enteritidis* cells grown at different pH levels

Partial mass spectra of polar lipid extracts of *S. enteritidis* strain 226 405 grown at different pH values and a medium control are illustrated in Fig. 6.3. The medium control illustrated was at pH 9.45. The assignment of the peaks as phosphatidylethanolamines (PE), phosphatidylcholines (PC) and sphingomyelin (SM) is shown, and the fatty acid composition for each peak is also indicated (Fig. 6.3).

- The major phospholipid species detected were phosphatidylethanolamines (PE) and phosphatidylcholines (PC).
- The proportion of phosphatidylethanolamines appeared to fall at pH 4.
- The medium contained predominantly phosphatidylcholines and sphingomyelin (SM), in similar proportions to the pH 4.35 cells.
- The phospholipid composition of the media controls was unaffected by pH (data not shown).

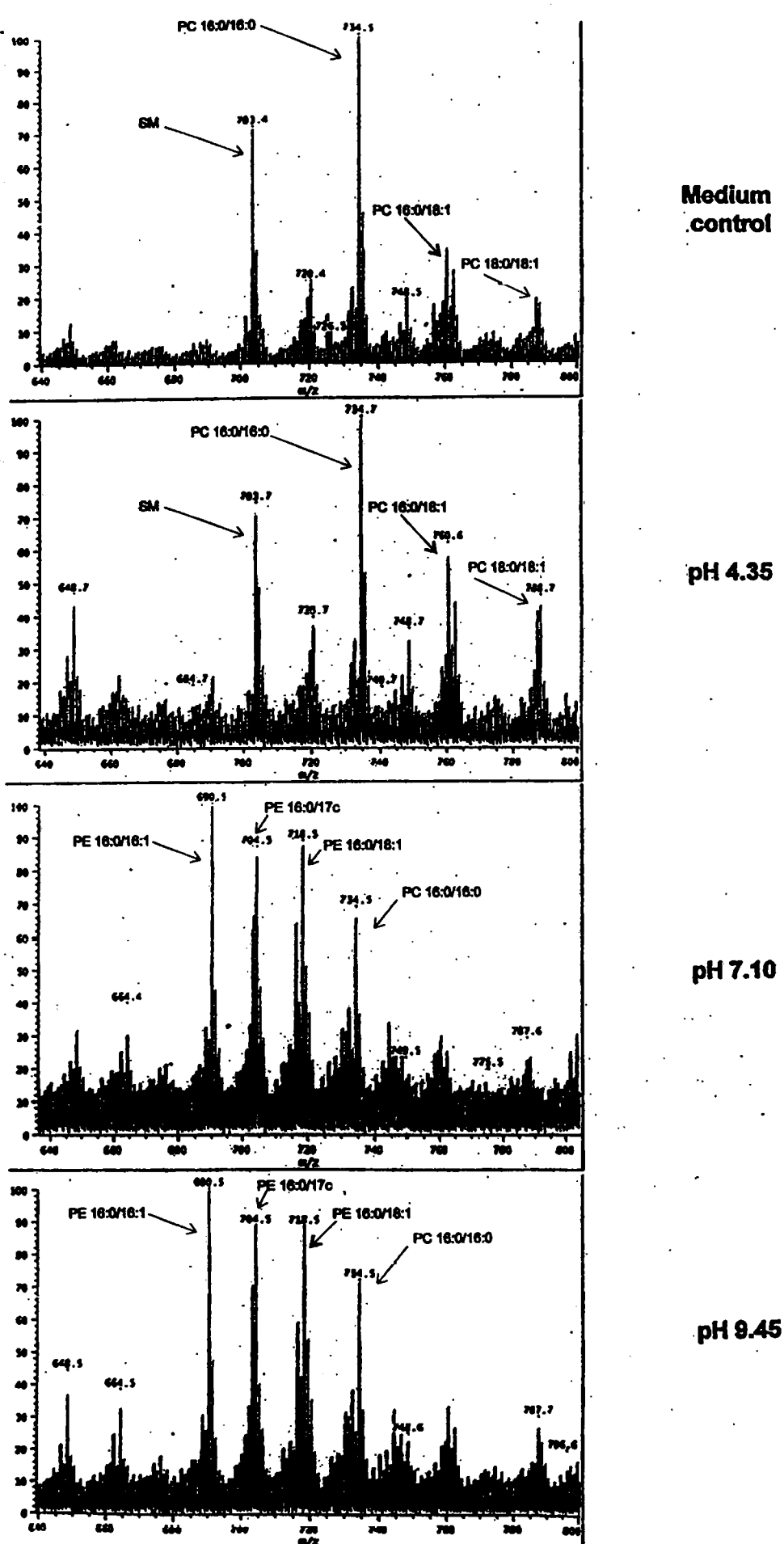


Figure 6.3 Partial mass spectra of polar lipid extracts of *S. enteritidis* PT4 grown at pH 4.35, pH 7.10 or pH 9.45 and a medium control

6.4 Discussion

In nature, bacteria are limited in their growth by the availability of essential metabolites and by the constraints imposed by hostile environmental factors. Extremes of pH are frequently amongst the hostile factors they experience (**Sections 1.11, 1.12 and 4.14**). The ability of *S. enteritidis* PT4 to replicate at low pH was shown to be impaired (**Table 3.1**), whilst the tolerance and virulence of these cells was enhanced (**Chapters 4 and 5**). These observations are consequences of the adaptation responses of *S. enteritidis* PT4 to external pH. In this study, the proteome and phospholipid content of cultures grown at different pH values was compared to identify some of the changes which take place at the molecular level in response to external pH.

These environmentally regulated processes which perform protective functions against adverse pH contribute to the 'stress response'. 'Stress response' is a general term and does not differentiate the different levels of damage which may be inflicted by pH extremes. The responses to pH include:-

1. adaptation, where changes in the bacteria optimise the ability of the cells to survive and grow under the prevailing adverse conditions (**Section 4.1.2**),
2. habituation and tolerance responses, where short-term exposure to mild pH increases the ability of the cells to survive subsequent, more extreme challenges, and
3. environmental shock, where the pH is bactericidal and it is the survival time which is considered.

An ability to modulate cell functions and structure to provide protection against environmental stresses will enhance the persistence and competitiveness of bacteria by enabling them to survive or continue limited replication in a wide range of ecological niches. The adaptation mechanisms of *S. enteritidis* PT4 to extremes of pH were investigated in this study, using continuous culture techniques because of the unique advantages provided by this approach. For example, the growth rate and other environmental conditions can be maintained constant whilst the pH is varied (**Section 3.1.4**). However, as pH affects other

environmental parameters it is impossible to study the effect of this parameter in isolation; this aspect is discussed further in **Chapter 7**.

The responses of *S. enteritidis* PT4 to growth at extremes of pH values are both specific and general in nature. The functions of the proteins modified during pH adaptation will determine whether any overlap occurs with other stress responses. The non-specific nature of some of the pH mechanisms was demonstrated in **Chapter 4**, e.g. cells grown at low pH had some cross-protection against a range of other stresses. It is likely that the damage induced by growth at the limits of pH causes a general disturbance to the metabolism of the cell, since the activity of key enzymes may be affected (**Sections 6.1.1 and 6.1.7**).

Investigations into the habituation/acid tolerance responses of *S. enteritidis* and *S. typhimurium* (**Sections 4.1.4 and 4.1.5**) have demonstrated a requirement for protein synthesis (Foster and Hall 1990, 1991; Foster 1991; Humphrey *et al.* 1993a). Modulation of protein expression is also a requirement for survival of pH shock (Foster 1991; Lin *et al.* 1995; Slonczewski and Foster 1996). Even though a role for protein synthesis in pH tolerance responses and survival has been identified, the protein patterns obtained from *S. enteritidis* PT4 cells grown at different pH levels, in this study were very similar (**Fig. 6.2**). The majority of the differences were quantitative, with very few novel proteins being observed (**Table 6.5**). Additional novel proteins may exist amongst the faint spots obtained in each of the protein maps, but these have not been considered in this investigation.

The total number of spots observed, using the methodology described (**Sections 6.2.3-6.2.6**) was approximately 200 at each pH level. However, the number of high intensity spots decreased slightly in cells grown at the limits of pH (**Table 6.4**), indicating that repression of protein expression as well as up-regulation or novel expression occurs in response to hostile pH. Cells may close down all non-essential functions in order to divert energy to protection processes, such as the production of stress proteins, and to transport systems which maintain a

functional intracellular pH. Synthesis of a limited number of apparently novel proteins was observed during growth at each of the extreme pH values (Fig. 6.2 and Table 6.5). These proteins may be novel or may be expressed in the other cultures, but at levels below the detection threshold of the gel loading and staining methods used.

The number of proteins which were regulated in response to acidic growth was lower than those regulated in cells growing at alkaline pH (i.e. 16 and 32 proteins respectively). Interestingly, only about half of the regulated proteins were up-regulated, with the other half being suppressed. The proportion of up-regulated proteins was slightly higher in acid grown cells (58%), than in alkaline cultures (46%). This confirms that the responses to high and low pH differ in *S. enteritidis* PT4.

Identifying and defining the role of stress proteins is complicated by the production of many of them as “house-keeper” proteins, and accounts for the predominantly quantitative variations between the protein profiles of cultures grown at different pH levels. Very low numbers of novel proteins were observed, one at low pH and four in the high pH cultures. Four novel proteins were also observed within the cells grown at neutrality (Table 6.5). The production of heat shock proteins in *E. coli* was discovered independently by two groups of researchers, who monitored the synthesis of individual proteins following a temperature increase using one or two-dimensional gels (Lemaux *et al.* 1978; Tamamori *et al.* 1978). They observed that approximately 20 proteins were temperature responsive, but most of them were not novel, but were required for normal cell growth.

Sixteen proteins were regulated in response to growth at pH 4.35, and this was similar to the number observed in *S. typhimurium* when tolerance to acid was induced. The expression of at least 18 proteins changed in response to a pH shift from 7.6-5.8 when the acid tolerance response (ATR) was induced in *S. typhimurium*, 12 of which were up-regulated and 6 suppressed (Foster and Hall 1990). The proportion of up-regulated proteins visualised in *S. typhimurium*

was 67%, which is slightly higher than the value of 58% determined in this study for acid-grown cells of *S. enteritidis* PT4. A slightly larger number of proteins were up-regulated in *S. typhimurium* following a pH fall from pH 7 to pH 5 than when growth was maintained at pH 5 (19 and 16 proteins, respectively) (Hickey and Hirshfield 1990). Again the number of proteins up-regulated in the adaptation of *S. typhimurium* to growth at pH 5 was similar to the value observed for *S. enteritidis* PT4 at pH 4.35 in this study (16 and 10 proteins, respectively). This may indicate that the responses to low pH are similar in *S. typhimurium* and *S. enteritidis*, although the identity and function of the proteins are still to be determined.

When *S. typhimurium* was exposed to acid shock the number of proteins which had altered expression increased to 52 (Foster 1991). These observations using *S. typhimurium* suggest that the adaptation response of *S. enteritidis* PT4 to growth at pH 4.35 may be closer to the induction of ATR, than to that of acid shock. This would be expected as the cells are able to adapt and grow at pH 4.35, although it is at the limit of their growth range and, consequently, represents a stringent test.

Good resolution was obtained using the 2D gel technique, and many proteins were visualised. However, one of the major limitations experienced in this study was the software used for comparing the protein maps. It was not possible to perform a direct comparison of more than two gels at any one time. This made estimations of the variability both within and between different chemostat cultures difficult. Experimental variability was assessed by comparing three replicate samples removed from the chemostats on different days. Incorrect assignment of spot numbers occurred with the software package making duplicate spots appear as different proteins. A greater number of errors occurred when the intensity of the spots to be counted was reduced, and it was necessary to confirm the findings visually.

A range of proteins were observed which varied in intensity with the growth pH; some of these are listed in Table 6.6. To date seven spots have been blotted

and used for sequencing (Tables 6.7 - 6.9). These protein spots were selected for more detailed investigation as they were expressed in relatively large quantities. The minimum protein level required for Edman sequencing is high, and the loading is limited to about 5-6 blot samples. Therefore, each blot must contain a large quantity of the protein. The sensitivity levels for peptide detection using mass spectrophotometric assays and peptide mass fingerprinting is much better (James 1997), but access to this technology was not available within this investigation.

One of the proteins, SES 2, was not sequenced due to a problem with the sequencer, and the sample was lost. The N-terminus of protein SES 6 was blocked, possibly because of post-translational modifications of the N-terminus, so again no sequence was obtained. Protein SES 7 was novel with no homologue matches being obtained in the Swiss-prot database. The detection of novel sequences is common. Various estimations of the ratio of novel open reading frames which cannot be assigned a function to those which can, based on homology searching are available. Over 20% of the proteins isolated in a study of the well-characterised *S. typhimurium* lacked homologues in the databases (Qi *et al.* 1996). A slightly higher value of between 40-60% was estimated in a review of proteome studies (James 1997).

The best matches with proteins in the database were obtained for spots SES 1 and SES 5. As expected, the most related proteins were from *S. typhimurium* and *E. coli*. The experimentally-determined values for the MW and pI values for these spots were in good agreement with the relevant *S. typhimurium* or *E. coli* protein, again providing further evidence for the validity of the identification. The match between SES 1 and the homologue GroEL is very good; 15 amino acids overlap and their positions in the complete protein are from residue 1 at the N-terminus (Tables 6.8 and 6.9).

GroEL has long been recognised as a heat shock protein, and it is the bacterial member of the hsp 60 family. Expression of GroEL was increased in cells growing at the limits of the pH range, with the highest expression being by cells

grown under alkaline conditions. *groEL* was one of the genes of *S. typhimurium* which was shown to be influenced by pH (Foster and Hall 1991). The role of GroEL in the correct folding, translocation and assembly of polypeptides must become more important during pH stress. This will, in part, be associated with the production of stress proteins.

The up-regulation of GroEL in response to a range of stresses in a variety of species has been demonstrated. These studies include the induction of GroEL following:-

1. alkaline conditions in *Campylobacter jejuni* (Wu *et al.* 1994),
2. a pH fall from pH 7.2 to pH 6.0 in *Porphyromonas gingivalis* (Vayssier *et al.* 1994),
3. 0.08% (w/v) bile salts, pH 4.8 or 50°C in *Enterococcus faecalis* (Flahaut *et al.* 1996),
4. high levels of ethanol in *Zymomonas mobilis* (Barbosa *et al.* 1994).

It is generally accepted that GroEL is not specific to heat shock and can be activated in response to a range of stresses (Welch 1993). However, no induction of GroEL was observed in *Bacillus subtilis* following salt or oxidative stress or glucose starvation and the researchers determined it be specifically responsive to heat shock (Volker *et al.* 1994). This was a surprising result as starvation and salt stress have both been shown to elevate the levels of GroEL in *E. coli* (Jenkins *et al.* 1991; Meury and Kohiyama 1991), and the studies listed above show that GroEL is not heat specific in a range of other bacterial species.

Enolase is involved in glycolysis in the sequence of reactions which converts glucose to pyruvate (frequently called the Embden-Meyerhof pathway) and catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate (PEP) which is a high energy phosphate compound (Lehninger 1975). The reaction is shown below:

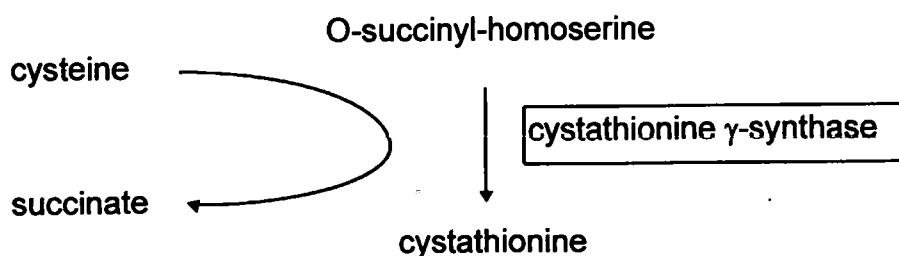


The match of SES 5 to the homologue enolase from *E. coli* was again close, with 15 amino acids overlapping from residue 1 (Tables 6.8 and 6.9). Enolase is

central to glucose metabolism and is common to different pathways. The phosphate is subsequently removed from PEP to form ATP and pyruvate; the net yield of glycolysis is two ATP molecules per molecule of glucose, consequently, enolase is involved in energy production. The expression of enolase was increased in *S. enteritidis* PT4 growing at pH 4.35. This may indicate that cells growing in acid conditions increase the activity of the PEP-phosphotransferase (PTS) transport system to extrude H^+ ions to maintain a functional intracellular pH. The major systems for controlling proton flow in and out of the cell are located in the cytoplasmic membrane and have been frequently shown to be affected by pH (Olson 1993). Alternatively, cells growing at low pH may generally have a higher energy requirement.

SES 4 was homologous to a precursor of the leu/ileu/val binding protein from *E. coli*, with a 12 amino acid overlap from position 24 from the N-terminal end, giving a percentage identification of 92% (Tables 6.8 and 6.9). This protein was expressed during growth at all pH values, but was up-regulated at low pH. The leu/ileu/val binding protein is sited within the periplasmic space and is responsible for interactions with membrane-bound proteins. It is an essential component of the high affinity active transport system for branched aliphatic amino acids in *E. coli* (Sack *et al.* 1989a, b), and it may act to regulate the internal level of target amino acids. Cells growing at low pH may use the decarboxylation of amino acids to increase their intracellular pH or, alternatively, the metabolism of cells at low pH may require the transport system for these amino acids to be more efficient.

Homologues were also identified to spot SES 3 which was suppressed in the cells grown at pH 9.45, although the matches were not close. Spot SES 3 had a region of overlap with cystathionine gamma synthase from the less closely related organism, *Haemophilus influenzae*. This enzyme catalyzes the reaction:



in the pathway which converts homoserine into the essential amino acid methionine. The overlap began at position 218 and only 8 amino acids matched, giving a percentage identification of only 62.5%, which was too low to be accepted. This suggests that the spot may have contained two proteins leading to incorrect sequencing and identification. The samples need to be re-run on gels with a smaller pH range which would enhance the separation of the proteins and confirm if cross-contamination was likely. This methodology would improve the isolation of pure protein samples.

The phospholipid content of the cells varied with growth pH (Fig. 6.3), with the proportion of phosphatidylethanolamines being lower in cells grown at pH 4.35. The phospholipid composition of cells grown at pH 7.10 and pH 9.45 were indistinguishable, suggesting that major changes in the phospholipid composition of the outer membrane are not required for *S. enteritidis* PT4 to grow under alkaline conditions. High levels of phosphatidylcholines were present in the medium, presumably due to the mucin content, and the mass spectra of the media controls were similar to those of cells grown at low pH (Fig. 6.3). It may be that pre-formed phosphatidylcholines are being incorporated into the cell membranes of cells growing at low pH directly from the mucin content of the medium. This may be a method for reducing the energy requirements of cells growing under these stringent conditions. No variations were observed in the levels of phosphatidylcholines in the medium at different pH levels (data not shown). Therefore, the changes observed with growth pH were not just reflective of the solubility or stability of mucin within the medium.

The cell membrane forms a permeable barrier against the external environment. Changes in the phospholipid composition will influence the activities of the large number of proteins which reside within this layer. These activities include transport, energy transduction and environmental sensing (Dowhan 1997), all of which would be expected to have a function in environmental adaptation. The phospholipid composition of pH 4.35 cells differed from that of cells grown at neutrality or pH 9.45. This again supports the theory that the responses to low

and high pH differ. Ion transport across the membrane is dependent upon the external pH of the cell and the activity of specific transport systems within the membrane. Changes in the phospholipid composition in response to environmental signals may enhance specific transport systems and assist environmental sensing.

pH 4.35 cells demonstrated an interesting ultrastructure when viewed by electron microscopy (**Fig. 3.1**). The protoplast appeared to have shrunk away from the cell envelope enlarging the periplasmic space. A number of processes that are vital to the growth and viability of Gram-negative bacteria occur within the periplasm. The periplasm is dynamic, and capable of regulation to compensate for changes in the internal and external environments which surround it. Also the architecture of the cell wall was more convoluted than in pH 7 or 9 cells (**Fig. 3.1**), indicating a possible role for cell surface structure and composition in acid adaptation by *S. enteritidis* PT4.

The mechanisms used by bacteria to sense and respond to external pH are still not fully understood. However, proteins which are regulated in response to external pH are being identified and potential functions ascribed. It is necessary to study the total set and quantity of proteins expressed in response to environmental and *in vivo* conditions to identify those which are environmentally regulated and which may have roles in survival and virulence. The adaptation responses of *S. enteritidis* PT4 to growth at the limits of the pH range involved the increased synthesis of some stress proteins and the concomitant suppression of other proteins and the expression of a very limited number of novel proteins. This suggests that adaptation to environmental pH is achieved by the regulation of 'in situ' bacterial processes. The adaptation responses of *S. enteritidis* may be similar to the inducible acid tolerance response described in *S. typhimurium* (Foster and Hall 1990).

The stress proteins are essential for the homeostasis of the cell; their roles in transport systems, and the folding and translocation of proteins become more important following a stress stimulus. This central role explains why stress

proteins are amongst the most conserved structures of living cells. Human and bacterial stress proteins also show an homology as high as 50%. This structural similarity links the immune responses against bacterial infections with a number of autoimmune diseases e.g. reactive arthritis (Lamb and Young 1990). GroEL is frequently up-regulated in response to stress in bacteria, it is highly conserved in nature and is a major target for the immune response during infection (Jindal *et al.* 1994).

In summary, protein expression was regulated in response to the growth pH of *S. enteritidis* PT4. Growth pH induced both up- and down- regulation of characteristic proteins. Acid grown cells had a higher proportion of up-regulated proteins than cells grown at alkaline pH. Growth at low pH stimulated the up-regulation of homologues to a precursor of the leu/ileu/val binding protein and enolase, when compared to cells grown at neutrality or pH 9.45. GroEL was also up-regulated in acid cells when compared to pH 7.10 cells, but the level of expression was further increased by cells grown at pH 9.45.

The phospholipid composition of pH 7.10 and pH 9.45 cells was similar, but differed from that of pH 4.35 cells. The proportion of phosphatidylethanolamines was lower in pH 4.35 cells, where the phospholipid composition was similar to that of the medium.

Chapter 7

General Discussion and Future Investigations

The reasons for the increased prevalence of *S. enteritidis* observed since the mid 1980's remain unclear. It is difficult to explain why this organism should become a problem in many areas of the world at the same time, and why the prevalence and causative phage type should vary globally. A proportion of the variations will be attributable to the surveillance and reporting procedures of different countries and also to different "farm to fork" practices. However, some of the variation may be a consequence of the virulence and tolerance of the strains which have contaminated the human food chain.

A number of key findings were made in this study that contribute to our understanding of the way in which *S. enteritidis* PT4 responds to external conditions, particularly pH, and of the relevance of these responses to the virulence and tolerance of strains. These findings will be discussed briefly below and their implications for future investigations will be highlighted (shown bulleted).

This study demonstrated for the first time that *S. enteritidis* PT4 was able to sustain replication under a wider range of environmental stresses when compared with other serotypes of *Salmonella*. In particular, PT4 and PT13a isolates had an enhanced tolerance of extremes of pH and temperature. In addition, *S. typhimurium* and *S. enteritidis* generally had faster growth rates when compared with other salmonellae. *Salmonella* pathogenicity is a multifactorial property, but requires the pathogen to be able to sustain growth in the complex, dynamic conditions of the 'environment', the food chain and a host. In addition, the occurrence of monocultures of bacteria in nature are rare events, and in most instances the pathogen will be in competition with many other microbes.

Therefore, the greater resistance to environmental extremes and the potential for faster replication demonstrated by *S. enteritidis* PT4 would be expected to enhance persistence and competitive ability and consequently pathogenicity. These properties may have contributed to the high incidence of *S. enteritidis* PT4 over the last decade. Some possible strategies to expand this work in future investigations are listed below.

- Future studies to screen the stress tolerance and growth rates of clinical isolates may indicate the contribution of these properties to the success of *S. enteritidis* PT4 as a human pathogen. Also, by profiling the growth rates and stress resistance of strains displaying different levels of pathogenicity, the interdependency of these factors and virulence could be identified.
- Mixed culture competitive growth studies at hostile pH values would indicate if *S. enteritidis* PT4 is able to out-compete other phage types, serotypes or bacterial species under extremes of pH. The competitive ability of *S. enteritidis* under a range of conditions with a relevance to host or food production and processing environments (e.g. extremes of pH and temperature, nutrient availability, oxygen availability, the presence of natural or artificial inhibitors or preservatives) could be determined in a chemostat.
- Gel-stabilised multiple gradient systems would enable the rapid screening of the effect of pH in conjunction with other environmental parameters (Peters *et al.* 1991; Thomas and Wimpenny 1996). Combinations of factors which induced the most significant responses could be studied in greater depth using growth studies in chemostats. Additional information would be gained by combining knowledge of the effect of individual parameters with that derived from combined factor experiments. These studies could provide valuable information on the importance of stress tolerance in determining the prevalence of specific serotypes and phage types of *Salmonella*. In addition, factors which act synergistically would be highlighted and this information would be of use in the development of food processing techniques

- Exposure to sequential stresses would demonstrate the potential and rate at which *S. enteritidis* cells adapt to the adverse conditions to which they are repeatedly exposed. This would indicate if *S. enteritidis* could be 'trained' to become more tolerant by pulses of stress factors. These types of experiments could also be used to measure the rate at which mutants with enhanced resistance are produced. This would provide valuable information for consideration in the risk assessment of food processing techniques.
- It is essential that the observations made in monocultures are extended and the effects of interactions with other bacteria or host cells are included when considering the immensely complicated interactions between *S. enteritidis* and its environment. Tolerance of acid pH in pure cultures does not always correlate with mixed culture situations, e.g. in a mixed culture of oral bacteria *Veillonella* spp. grew at pH 5 although this was not possible in pure culture (McKee *et al.* 1984). Animal or *in vitro* models could be used to determine the ability of *S. enteritidis* PT4 to compete in specific environments.
- The persistence of *S. enteritidis* PT4 could be compared with that of other enteric pathogens by using *in vitro* models, e.g. the three stage model of the colon (Gibson *et al.* 1993). However, extrapolation between laboratory models and host environments would still be limited due to the large number of interactions which are at work.
- Chickens and laying hens could be used to compare the competitive ability of *S. enteritidis* PT4 against other salmonellae within *in vivo* environments. By challenging birds with defined inocula of *S. enteritidis* PT4 in combination with other bacteria the invasiveness and persistence within different tissues and the frequency of egg contamination demonstrated by the component bacteria could be determined.

This thesis provides some of the first demonstrations that both *S. enteritidis* PT4 and *S. typhimurium* have considerable resistance to aerosolisation. *S. enteritidis* survives for extensive periods in disused animal houses, in some cases for up to

a year, (Davies and Wray 1996), and on surfaces after dissemination, e.g. following whisking of a contaminated product (Humphrey *et al.* 1994). These environmental observations support the findings that *S. enteritidis* PT4 has considerable resistance to air drying.

The survival times determined in this study exceeded those published for recognised respiratory pathogens such as *Legionella pneumophila* and *Mycobacterium tuberculosis*. The infective dose for aerosolised *S. enteritidis* PT4 is lower than for oral or intra-peritoneal challenge in poultry (Baskerville *et al.* 1992; Chart *et al.* 1992; Humphrey *et al.* 1996). Also, when two isolates of *S. enteritidis* PT4, with varying virulence in mice and invasiveness in laying hens, were compared, the more virulent strain survived longer in aerosols (Humphrey *et al.* 1996). Therefore, maintaining viability whilst airborne may have a significant role in the transmission of *S. enteritidis* PT4 and should be considered further. Possible future studies are bulleted below.

- Measuring the number of viable *S. enteritidis* cells in air samples obtained at different stages between the farm and the kitchen during the processing of contaminated carcasses would confirm the potential for airborne dissemination and transmission of *S. enteritidis*. Infection of wild mice or birds can cause cross-contamination between different sites and, consequently, aerosol infection of these vectors will contribute to the persistence of *S. enteritidis* PT4.
- Screening a range of clinical isolates for aerosol tolerance may identify any correlation between enhanced aerosol resistance, and human disease.

Hostile pH at both acidic and alkaline values has a major relevance to the habitats of enteric pathogens in food, hosts and the environment. The influence of pH is therefore of considerable interest and many studies have looked at different aspects of bacterial responses to pH challenges. This thesis includes novel studies of *S. enteritidis* PT4 grown in continuous culture at different pH values. Most other studies of *S. enteritidis* PT4 have used batch cultures where

environmental conditions are ill-defined. In the chemostat, bacteria are grown under controlled conditions at a rate determined by the availability of an essential nutrient, and this results in a homogeneous culture (Tempest 1970a). Repeat chemostat runs result in essentially identical cultures, enabling large volumes of culture to be harvested for numerous studies and repeat determinations. Individual environmental parameters can be controlled or varied as required allowing the cause-and-effect relationship between growth pH and the survival and pathogenicity of *S. enteritidis* PT4 to be established.

As pH has a considerable effect on other parameters it is impossible to study the primary effect of this factor in isolation. Many secondary effects of pH also influence the overall state of bacterial cells. It is likely that pH-induced stress causes a general disturbance of cell metabolism, since the activity of key enzymes are frequently pH dependent. Solubility, ionisation state and redox potential are all related to pH e.g. there is a 10^{23} difference in the solubility of Fe(III) between pH 2.0 and pH 10.0 (Olson 1993). This causes additional pressures on the bacteria i.e. high concentrations of iron must be tolerated at low pH levels, whereas efficient retrieval systems are required at higher pH levels. pH also regulates anaerobic metabolism (Slonczewski and Foster 1996), as under anaerobic atmospheres the transmembrane electrical potential is lower and is further reduced as the pH decreases (Kashket 1981). Although problems in the investigation of pH have been identified, many of these were addressed by using continuous culture. Factors such as temperature, availability of oxygen, levels of metabolic products and cell density were maintained constant during the culture of cells throughout this investigation as variations within these parameters can confound the identification of the effects of pH.

An important finding of this study was that growth pH influenced the survival and virulence of *S. enteritidis* PT4. Growth at low pH induced cross-protection against a range of stress factors, and increased the virulence of cells in a mouse model. In contrast, adaptation to alkaline growth induced sensitivity to acid, low temperature, crystal violet and NaCl and did not modify the virulence of the cells. A correlation between the tolerance and virulence of *S. enteritidis* PT4 strains

has been postulated, since isolates of *S. enteritidis* PT4 with enhanced heat and acid tolerance were more virulent in mice and invasive in laying hens, although no significant differences were observed in chicks (Humphrey *et al.* 1996). This hypothesis was supported in this study with the more stress-tolerant pH 4.35 cells also being the most virulent in mice. Cell properties which may have contributed to the altered virulence and resistance in response to growth pH are outlined below.

A significant observation of this study was that *S. enteritidis* PT4 cells growing at the limits of the pH range were predominantly devoid of type 1 fimbriae (SEF 21) and only a small proportion expressed flagella; however, a decrease in the proportion of cells expressing flagella was observed in continuous cultures, generally. As the cells grown at pH 4.35 were the most virulent when assayed using intra-peritoneal challenge of mice, and the proportion of cells expressing SEF 21 was low, it suggests that SEF 21 do not have a major role in the tissue invasion of mice by *S. enteritidis* PT4, and their function in disease remains a matter of conjecture. The proportion of bacteria expressing SEF 21 was not determined in cells reisolated from infected tissues.

The role of flagella in the pathogenesis of *S. enteritidis* PT4 is also still to be determined. The absence of flagella on *S. enteritidis* PT4 cells did not reduce their pathogenicity in the mouse model; however, the proportion of bacteria expressing flagella increased within spleen tissue, indicating a potential role in tissue invasion. The effect of environmental conditions on the expression of flagella and fimbriae suggests that their roles may be related to survival, being switched on and off rapidly in response to environmental shifts. A more detailed investigation of the expression of specific surface structures by *S. enteritidis* PT4 during different stages of infection would be of value.

- Funding was secured from the Department of Health based on the observations above, to establish the role of specific surface structures in the pathogenicity and persistence of *S. enteritidis* PT4. This study compared the pathogenicity of deletion mutants lacking specific surface structures, either

individually, or in combinations. The effect of environmental conditions on the expression of specific structures was also determined (in collaboration with the Central Veterinary Laboratory).

The present study did not demonstrate a major role for the 38MDa plasmid in the virulence of a clinical isolate of *S. enteritidis* PT4 in mice following intra-peritoneal challenge. However, the virulence of strain 226 405 in the mouse was generally low when compared to other *S. enteritidis* PT4 isolates. This suggests that this strain may be compromised in some way and is unable to express the plasmid 'virulence' genes. The role of this plasmid in salmonellosis is still not fully elucidated and plasmid negative strains are isolated at a low frequency from infected humans and poultry (Threlfall *et al.* 1994). This demonstrates that the plasmid genes are not essential for virulence, but act to increase pathogenicity. Future studies (suggested below) are required to confirm if the plasmid does not have a role in the pathogenicity of this strain.

- Measuring the level of carriage of the 38MDa plasmid in bacteria retrieved from sites within infected mice would confirm if this strain of *S. enteritidis* PT4 has extraintestinal virulence without the information carried on this plasmid.
- It would also be pertinent to compare the effect of plasmid carriage on the virulence of this isolate following oral challenge, either by using invasion kinetics as a measure of extraintestinal colonisation or by extending the time period of the test to observe mortalities. It would also be interesting to ascertain if this strain is able to express the 38MDa plasmid genes.

S. enteritidis PT4 strains demonstrate a wide range of virulence levels in animal models (Table 5.8) (Humphrey *et al.* 1995, 1996). The clinical isolate used in this investigation generally did not cause disease symptoms in mice within 14 days following oral challenge with up to 10^7 cfu.

- It would be interesting to screen clinical isolates to determine the proportion which would be categorised as being of low virulence when tested in the

mouse model. It may be that the less invasive strains can persist in natural vectors e.g. wild mice and birds and provide continual cross-contamination of the human food chain.

- Studies to identify the characteristics which discriminate between strains of *S. enteritidis* PT4 which demonstrate extremes of pathogenicity would provide indicators of virulence determinants.

This thesis includes one the first studies of the phospholipid content of *S. enteritidis* PT4. The phospholipid composition of cells growing at pH 7.10 and pH 9.45 were similar. However, when grown at low pH, *S. enteritidis* PT4 appeared to incorporate phospholipids directly from the medium. This may be a mechanism for regulating specific transport systems through the cell membrane, or it may be to reduce the energy required for phospholipid synthesis. The maximum growth rate of cells at pH 4.35 was reduced when compared with cells at pH 7.10 or pH 9.45 (Table 3.1), again suggesting higher energy demands for cells growing at low pH.

pH adaptation involved the regulation of 'normal' cellular functions and very few novel proteins were expressed in response to changes in growth pH. The levels of homologues to enolase, GroEL and a precursor of the leu/ileu/val binding protein were up-regulated at pH 4.35. Enolase is involved in energy metabolism and the leu/ileu/val binding protein is associated with amino-acid transport which could be a component of the mechanisms used for intracellular pH regulation.

The phospholipid composition of alkaline-grown cells was indistinguishable from those grown at pH 7.10, but had higher levels of phosphatidylethanolamines than cells growing at low pH, or than were present in the medium. Phosphatidylethanolamines have been shown to have properties which are required for membrane protein assembly. Changes in the phospholipid composition can alter the fluidity of the membrane which would affect the secretion of extracellular products etc. The chaperone protein GroEL was also up-regulated in cells growing at pH 9.45. This protein has been demonstrated to

be a general stress protein, being up-regulated in response to a range of stresses in a variety of bacterial species. However, the levels of enolase and the leu/ileu/val binding protein were the same as in cells grown at neutrality.

Throughout this investigation pH adaptation resulted in changes to the cell surface. The following all demonstrated some dependence upon growth pH:- the tolerance to surface active agents, the expression of surface structures, and the phospholipid composition. Consequently

- a detailed investigation of the composition and structure of the cell surface of a range of isolates grown under different conditions would provide valuable information on their environmental responsiveness and possibly their functions in survival and pathogenicity.

Determining the global response of *S. enteritidis* PT4 to environmental signals that regulate its pathogenicity is essential to the understanding of salmonellosis. Transcriptional regulation by a cascade of 'two-component' systems enables pathogenic bacteria to express their survival and virulence determinants in response to a broad spectrum of external stimuli (Soncini and Groisman 1996). It has been estimated that *S. typhimurium* has more than 50 different 'two-component' systems (Stock *et al.* 1989).

- A comparison of the expression of proteins and postulated virulence factors by a population of *S. enteritidis* PT4 cells used to challenge animals with those expressed following recovery, from different tissues during infection would establish the importance of these factors in the pathogenicity of *S. enteritidis* PT4. Bacteria would need to be recovered without replication or protein synthesis or denaturation.
- It would also be of value to identify the characteristics of *S. enteritidis* PT4 cells when growing in food products which are frequently implicated in disease e.g. in eggs, on poultry skin or within mayonnaise.

By understanding the processes by which *S. enteritidis* recognises and responds to the environment, it may be possible to identify novel targets for intervention strategies to interrupt these processes and prevent cell adaptation.

In summary, *S. enteritidis* PT4 was able to grow at extremes of pH which were inhibitory to most other salmonellae. The physiology and metabolism of *S. enteritidis* PT4 was altered during adaptation to growth at different pH values. The adaptive responses to high and low pH differed, in that cells growing at low pH appeared to have a greater energy requirement. These responses included changes in the proportion of cells expressing surface structures, the level of carriage of the 38MDa plasmid, the phospholipid composition and the expression of proteins involved in transport, energy production and chaperone processes. These changes modified the survival and virulence of *S. enteritidis* PT4. Growth at low pH enhanced the survival and virulence of cells, whilst growth under alkaline conditions increased the sensitivity of cells to acid and surface active agents and did not cause a significant change in the pathogenicity of the cells. In conclusion, growth pH influenced both the survival and the pathogenicity of *S. enteritidis* PT4 (clinical isolate 226 405).

This study has provided some new insights into the metabolic and physiological changes which contribute to the survival, persistence and virulence of *S. enteritidis* PT4 under a range of environmental conditions.

Chapter 8

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Publications

To date the following have been published from areas of research performed within this thesis:

McDermid, A.S., McKee, A.S., Dowsett, A.B. and Marsh, P.D. (1996) The effect of environmental pH on the physiology and surface structures of *Salmonella* serotype enteritidis phage type 4. *Journal of Medical Microbiology* **45**,1-7.

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McDermid,A.S, McKee,A.S, Williams,,A and Marsh,P.D. (1997) The influence of environmental pH on the physiology and pathogenicity of *Salmonella enteritidis* phage-type 4. *Salmonella and Salmonellosis International Symposium Proceedings*. 147-151.

Oral presentations have also been presented and abstracts published as listed below.

McDermid,A.S, McKee,A.S and Marsh,P.D. (1995) A comparison of the growth rates and ability to grown under extreme conditions of different serotypes and phage-types of *Salmonella*. PHLS Annual Scientific Conference.

McDermid,A.S, McKee,A.S and Marsh,P.D. (1995) Effect of environmental pH on the physiology of *Salmonella enteritidis*. *Society for General Microbiology*.